EFFECTS OF NICOTINE, STRESS, AND SEX ON BEHAVIORAL INDICES OF DEPRESSION AND ANXIETY IN RATS

by

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Abstract

Title of Thesis: Effects of Nicotine, Stress, and Sex on Behavioral and Biological Indices of Depression and Anxiety in Rats

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The health hazards of tobacco and the addictive effects of nicotine are well-established and well-known. However, these health hazards are especially pertinent upon our consideration of the military population, given the knowledge that tobacco use is more prevalent in the military as compared to civilians. Additionally, smoking status for military personnel is associated with perceived stress. These findings naturally merit questions of critical importance that affect the young Americans tasked with defending our nation: it may seem that there are potential benefits of nicotine and nicotine analogues that merit consideration and experimental analysis.

The present experiment used 64 male and female Sprague Dawley rats to examine the effects of nicotine with and without exposure to stressors. The dosage of nicotine was chosen to model effects of humans smoking ½ to 1 pack of cigarettes per day. To closely model the stress experienced during a military combat deployment, the experiment utilized a stress paradigm that models threat of attack to create a substantial stress response without physical harm. The behavioral measure used was open field activity (to assess general activity, depressive-like behavior, anxiety-like behavior).

Overall, results differed based on sex, stress, and time. At 10 days after nicotine administration, stressed female rats receiving nicotine displayed increased horizontal activity. In contrast, male rats that received nicotine did not display increased horizontal

activity until 20 days after drug administration. There were no significant effects of nicotine or stress on depressive-like or anxiety-like behaviors.

The present findings suggest that nicotine increases general activity for both sexes, with females displaying more activity sooner than males, and for mainly rats that were stressed. The experiment provides important implications for military service members operating under stressful deployment conditions: nicotine may serve to generally increase the activity-levels of service-members who are experiencing stress. Additionally, observed sex differences with regard to this effect suggest pertinent avenues of exploration in light of increasing interest in allowing female service-members entry into combat positions within the military.

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CHAPTER 1: Introduction

Worldwide approximately 1.3 billion people smoke cigarettes and almost half will

die prematurely from smoking-related illnesses (129; 130; 131). In the United States

(U.S), tobacco is the leading preventable cause of death (59). From 2005-2009, cigarette

smoking and exposure to tobacco smoke led to 480,000 premature deaths annually in the

U.S (118). Chronic health disease as a result of tobacco use accounts for 75% of

American healthcare spending (117). Specifically, cigarette smoking is the predominant

form of tobacco use and in the U.S, almost 20% of American adults use tobacco.

Additionally, cigarette smoking is more common in males than females; and is more

frequent among people with less education and lower socio-economic status (118). Of

critical importance for young Americans, is the fact that nearly all tobacco use begins in

childhood and adolescence. Among adults who had ever tried a cigarette, more than 80%

reported trying their first cigarette by the time they were 18 years of age (117).

With concern towards the young Americans tasked with defending the United

States, tobacco use is more prevalent in the military as compared to gender- and age-

matched civilians (72). According to the 2011 Department of Defense (DoD) Health

Related Behaviors Survey of Active Duty Military Personnel, total DoD cigarette

smoking prevalence was 24%. However, other research has indicated a higher rate of

smoking for military service members (Warriors), with prevalence estimates being

around 40% (91). The mean prevalence rate for smokeless tobacco (ST) users in the

military is about 9.4%, and the majority of ST users in the military are enlisted white

males (10).

Consequently, given the deleterious effects of smoking on health, there have been efforts at the policy level to reduce smoking in the military. Each branch of the armed forces has launched individual initiatives to curb smoking, and the DoD has implemented policy banning smoking from all DoD workplaces (72). However, while considering the disproportionately high usage of tobacco products in the military, one may wish to explore possible explanations for this critical military health issue.

To possibly address these concerns, research has shown that for Warriors, smoking status was associated with perceived levels of stress (115). Given the high prevalence of tobacco use in the military, and particularly under stressful conditions, important questions emerge: Are Warriors using tobacco and other nicotine products as a means to self-medicate under stressful conditions? And if so, does nicotine use actually decrease levels of stress-induced negative affective states? Before addressing these questions, it bears relevance to consider the relationships between smoking and stress, as well as other negative affective states (e.g., depression, anxiety) as a result of stress.

Smoking and Stress

The 1988 Report of the Surgeon General (SGR) <u>Nicotine Addiction</u> provides the earliest comprehensive summary on the relationship between smoking and negative affective states (116). Pertinent to the following discussion, the term *negative affect* is non-specific, and refers to a variety of aversive mood states, which include: anger, contempt, disgust, guilt, fear, and nervousness (76; 120). As indicated in the 1988 SGR, previous research has for many years suggested the occurrence of elevated smoking rates during different types of negative affective situations (11; 28; 70; 80; 85; 116). In particular, smoking has been associated with anxiety, aggression, neuroticism, and

suicide (23; 26; 37; 79; 108; 116). Additionally, recent research has strongly suggested

that smoking reduces negative affect in adolescents (75). With regard to the specificity of

negative affect, there has also been long-standing evidence that stress increases the

likelihood of smoking initiation, in particular for adolescents (49; 54; 116). Importantly,

however, the most direct evidence linking smoking to negative mood states came from

studies that utilized measures of subjective stress (68; 88; 116; 124;125).

Stress has been defined as the process by which environmental demands tax or

exceed the adaptive capacity of the organism (5). Acute and chronic stress are linked to

altered HPA (hypothalamic pituitary adrenal)-axis responding, disordered glucose

metabolism, increased catecholamine transmission, and other metabolic endocrine effects

(33). As outlined in the 1988 SGR, laboratory studies confirmed that smokers smoke

more during stressful situations (42; 102; 106; 116). Additionally, studies involving

Naval personnel have linked stress to smoking (17; 31).

More recently, the notion that negative affective states and smoking are related

have held true. As reported in the 2012 SGR Preventing Tobacco Use Among Youth and

Young Adults, smoking among young Americans is associated with higher levels of

negative affect as compared to non-smoking peers (88; 32; 43; 86; 117). Newer evidence

has also suggested that among adolescents, cigarette smoking appears to provide

immediate and reinforcing changes in both positive and negative moods (76; 75).

Interestingly, adolescents that expect to receive greater mood benefits, experience

increases in positive mood after smoking (30; 117). With regard to stress, studies have

found associations between stress and tobacco use among adolescent smokers (110; 117;

126).

Smoking and Depression

As summarized in the 2012 SGR, there is substantial evidence to support the notion that cigarette smoking is related to depression (117). Of the various forms of negative affect, depression is the most notable form that is particularly linked to smoking initiation (76). According to the Diagnostic and Statistical Manual of Mental Disorders (5th ed.; DSM-5; 2), major depressive disorder is primarily characterized by the presence of sad, empty, or irritable mood, while individual symptoms include markedly diminished interest; weight change; sleeping difficulty; psychomotor agitation or retardation; fatigue; feelings of worthlessness; difficulty concentrating; recurrent thoughts of death or suicidal ideation.

Research has demonstrated that depression and anxiety predict smoking initiation among youth (93; 117) and that smoking during adolescence increases the risk for development of mood and anxiety disorders (73; 117). Specifically with regard to depression, increased depressive symptoms predict elevated levels of smoking (3; 128; 117). Importantly, Audrain-McGovern et al. (3) involved an adolescent population, which suggests increased pertinence when considering the age-range for most Warriors. Taken together, the literature presents a bi-directional relationship between smoking and depression with regard to the stages of initiation and maintenance. While studies suggest that there is evidence for self-medication through smoking for depressed individuals, other research has suggested that smoking leads to depression as well (76).

Smoking and Anxiety

While there are multiple anxiety disorders (e.g.., specific phobia, social anxiety disorder, panic disorder, agoraphobia, generalized anxiety disorder) within the DSM-5,

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all are characterized by excessive fear and anxiety with related behavioral disturbances (2). As with depression, research has indicated that there is a role for anxiety in explaining smoking behavior (45; 73; 94; 93; 117). However, the relationship between smoking and anxiety has been difficult to ascertain with the available literature. With regard to smoking initiation, research findings are unclear. While it has been reported that adolescents with anxiety symptoms are more at risk to start smoking than adolescents without anxiety symptoms, other researchers have found no association (36; 76; 94). With regard to smoking maintenance, the findings are bi-directional (76).

Smoking and Sex Differences

The 2014 SGR tragically reported that over the past 50 years, the disease risks from smoking by women have risen to the point where they are now equal with men for lung cancer, chronic obstructive pulmonary disease, and cardiovascular diseases (118). While this finding is of critical importance, previous research has long suggested that there are sex differences with regard to smoking behavior (63). Specifically, it has been reported that females score higher on negative affect reduction from smoking; and that under stress, females are more likely to try additional cigarettes after the initial smoking experience (50; 70; 71; 109). In light of these reported sex differences with regard to smoking behavior as well as numerous other research domains, it comes as no surprise to learn that the National Institutes of Health now require the inclusion of males and females in all cell and animal studies (27).

Stress and the Military

As outlined earlier, stress has been linked to the use of various substances, including nicotine (58; 76). Considering the relationship between stress and tobacco use,

the military population provides a pertinent example of clinical interest for tobacco use given the distinctively stressful environment service members operate within (115).

More recently, Warriors have been exposed to stressful environments while conducting ongoing combat operations in support of the Global War on Terrorism (GWOT), which has included Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF). Specifically, the threat of death or serious injury is a particular psychological stressor inherent with combat deployments. During these combat deployments, Warriors have been frequently exposed to improvised explosive devices, rocket-propelled grenade attacks, and small arms fire (12). Research has indicated that these exposures to violent combat events, such as firefights, are the mechanisms mainly responsible for driving the psychological costs of these wars, including combat stress (24; 101). Directly related to the stress associated with threat of death, which is inherent in firefights and other combat events, it seems no surprise to learn that those who were involved in firefights more frequently, were at greater risk for suicidal ideation, depression, and Posttraumatic Stress Disorder (PTSD) as compared to those who were involved in fewer firefights (24).

Almost intuitively, research has found that combat exposure significantly predicts perceived stress (87). Given the likely association of perceived stress with PTSD and other stress-related disorders, it may be important to note that over 1.8 million U.S. Warriors have served in OIF and OEF, and the PTSD prevalence for these Veterans was recently estimated as ranging from 10% to 18% (81). Succinctly, the stress associated with threat of death during combat deployments has inarguably taken its psychological toll on Warriors.

Stress and Tobacco Use in the Military

Research has long established a link between stress and substance use (58). Warriors who report using tobacco to reduce stress, report significantly higher levels of stress than Warriors who do not use tobacco (115). In relation to this link between stress and smoking for Warriors, research has indicated a strong association between PTSD and high rates of smoking (51). More recently it has been reported that post-9/11 Veterans with PTSD maintain greater expectancies that smoking reduces negative affect than smokers without PTSD (19; 84). Furthermore, it has been reported that among Warriors, non-smokers initiate smoking during deployment; and that there is increased risk for smoking recidivism for those who have deployed as compared to those who have not deployed (111).

It seems well-established that for Warriors, there is a clear relationship between tobacco use and stress, as well as stress-induced negative affective states. However, aside from these findings, we return to the question posed above: Does tobacco actually decrease levels of stress-induced negative affective states? To respond to this question appropriately, it would seem necessary to explore the key ingredient within tobacco: nicotine.

Nicotine

Experimental research has long-established that nicotine (3-(1-methyl-2-pyrrolidinyl)-pyridine) is the most important component of the affect-modulating properties in tobacco (53; 55; 62; 95; 98; 69; 74; 105; 121; 116). Nicotine is a highly toxic liquid alkaloid found in several plant species that can exist in two enantiomeric forms, but in nature exists in the S-Shape, or levorotary form. The half-life of nicotine is

approximately two hours (62; 59). Biological theories have emphasized the reinforcing properties of nicotine in accounting for addiction (116). Furthermore, the resulting maintenance of tobacco use is especially driven by the psychopharmacological mechanisms involved with nicotine (59).

Specific psychopharmacological explanations for the use of nicotine in association with stress and negative affect are abundant (76). One may consider the Yerkes-Dodson curve of performance and emotional arousal, whereby the effect of nicotine seems to be especially powerful given its paradoxical ability to provide stimulation during fatigue, and relaxation during anxiety (135; 58). However, while smoking is perceived to reduce stress for individuals per self-reports, the data are mixed with regard to whether nicotine is in fact responsible for mood modulation in response to stressful stimuli (116).

Animal research may be an important domain in terms of growing the research in response to this research dilemma. Importantly, animal research has long been demonstrated to be a viable means of examining the effects of nicotine on appetitive behaviors in rats (56; 61). Animal research has reported that different stressors (e.g., footshock stress, food deprivation) increases nicotine intake in rats (16; 34; 76). Furthermore, the animal literature has suggested that there are important sex differences with regard to the effect of nicotine on behavioral indices of negative affect: female rats are more sensitive to the anxiolytic effect of nicotine as compared to male rats (25); unstressed female rats that received moderate doses of nicotine displayed less depressive-like behavior than unstressed female rats that received saline (90).

Given the increased pertinence of considering nicotine use in the military population, the purpose of the present study was to investigate the effects of nicotine and stress on behavioral indices of stress and depression by conducting an experiment with rats. Additionally, given the growing interest with integrating women into combat arms positions in the military, the effects of sex (male, female) was another variable of interest. Importantly, since the start of the War on Terror, almost 300,000 women have deployed in support of OIF and OEF; over 800 women have been wounded; over 130 have been killed. While laws prohibiting women from serving in combat units were repealed more than twenty years ago, it has been DoD policy to restrict women from particular units and military occupations. Recently, this policy has been under intense congressional review (18), and there have been recent efforts to academically explore the role of women in combat (35).

The experiment utilized behavioral indices of depression and anxiety. Behavioral measures included open-field activity (OFA) vertical activity (VA) and OFA center-time (CT) for depressive-like and anxiety-like behaviors respectively. A detailed description of each behavioral measure is provided further below in the Methods section.

The Value of Animal Models

Based on the available literature (51; 115; 19; 84), it is evident that usage of nicotine in Warriors is linked to stress and negative affect. However, there are several limitations within the available literature, which involve study design, measurement, and ethics. With regard to study design, current studies are mainly correlational, such that the literature cannot prove causality between nicotine use and stress or negative affect under stressful and non-stressful conditions. Additionally, correlational analyses are unable to

distinguish the effects of one variable from another. This key limitation in study design has been a common factor for the vast majority of research involving human subjects (75; 76). Specifically, with regard to smoking and stress, studies are mainly correlational; with regard to smoking and depression, the predominantly correlational literature suggests a bi-directional relationship; with regard to smoking and anxiety, the literature is unclear if a relationship even exists (76). With consideration towards measurement limitations, the majority of studies involve subjective measurements, which naturally limit interpretation of data which is ostensibly biased (76).

Finally, in light of ethical limitations in conducting a true experiment with adolescent humans to investigate whether or not nicotine use indeed buffers against stress (e.g., administering nicotine to non-smoking minors), an animal model provides a direct and ethical means to investigate causality, minimize confounding factors, and isolate variables of interest. Importantly, leading researchers have advocated for such animal models to help ascertain these important relationships (76). An animal model will provide the experimental control of key independent variables (i.e., nicotine/saline, stress/no stress, male/female), and dependent variables (i.e., anxiety-like and depressive-like behaviors).

CHAPTER 2: Overview and Specific Aims

The present experiment was designed to determine effects of nicotine, stress, and sex on behavioral indices of depression and anxiety in an animal model. There were two specific aims of this experiment: (1) to examine effects of nicotine on depressive-like and anxiety-like behavior under stressful and non-stressful conditions; (2) to determine whether there are sex differences with regard to the effects of stress and nicotine on depressive-like and anxiety-like behavior.

Hypotheses

The following are hypotheses according to each specific aim.

Specific Aim 1: To examine effects of nicotine on depressive-like and anxiety-like behaviors under stressful and non-stressful conditions in male and female rats.

Hypothesis 1a: Stressed rats will exhibit more depressive-like and anxiety-like behaviors as compared to unstressed rats.

Rationale: Previous research has indicated that the Warrior Stress Paradigm (WSP) not only increases biochemical markers of stress (8; 9; 82; 97; 114) but in combination with other stressors also increases depressive-like and anxiety-like behavioral responses in male and female rats (8; 132). However, the effect of the WSP alone (without other stressors) on behavioral responses specifically is yet to be ascertained, and the present experiment was designed to expand the literature in addressing this question.

Hypothesis 1b: Nicotine will attenuate depressive-like and anxiety-like behavior for stressed and non-stressed rats.

Rationale: Previous research has linked the use of nicotine to modulation of mood (14; 76; 75). This experiment will expand the literature by contributing experimental findings via use of animals.

Hypothesis 1c: Nicotine will be a protective factor against stress as indicated by lower levels of depressive-like and anxiety-like behaviors in stressed animals administered nicotine as compared to stressed animals administered saline.

Rationale: Previous literature has outlined the use of nicotine in the context of military-related stress (e.g., threat of death; environmental stress; 19; 115) and stress in general (58; 76). This experiment will expand the literature by providing experimental findings that incorporate the WSP.

Specific Aim 2: To determine whether there are sex differences with regard to effects of stress and nicotine on depressive-like and anxiety-like behavior.

Hypothesis 2a: Stressed male rats will display less depressive-like and anxiety-like behaviors as compared to stressed female rats.

Rationale: Previous literature has indicated that female rats are more sensitive to predatory and environmental stress as indicated by increased levels of depressive-like and anxiety-like behaviors (9; 132; 134).

Hypothesis 2b: Nicotine will produce greater attenuation of depressive-like and anxiety-like behaviors for female rats as compared to male rats.

Rationale: Previous research has provided preliminary evidence that for unstressed females, nicotine attenuates depressive-like behavior (90). This experiment will expand on these findings.

CHAPTER 3: Methods

To address the above hypotheses, the experiment was conducted as a 2 (saline, 6 mg nic/kg/day nicotine) x 2 (no stress, stress) x 2 (male, female) full factorial mixed design. This experimental design resulted in 8 experimental conditions. There were 8 subjects in each treatment condition (Table 1). The number of subjects per condition was based on previous research by the Grunberg Laboratory, which utilized a variety of independent and dependent variables similar to the present investigation (1; 46; 39). There were a total of 64 subjects, which were investigated in two separate counter-balanced cohorts of 32 subjects. Experimental procedures and environmental conditions were identical across the two cohorts (see Figure 2 for experimental timeline). Animal husbandry conditions, independent variables, dependent variables, experimental timeline, and data analytic strategy are explained below.

Animals and Housing

The subjects of the experiment consisted of 64 Sprague-Dawley rats from Charles River Laboratories (Wilmington, Massachusetts). To closely model the age range of young Warriors, the ages of subjects ranged from 51-55 days. While research indicates that adolescence for rats ends at 55 and 42 days for males and females, respectively (92; 113), a conservative age zone does not imply strict limitations on adolescent behavior of animals in the gray zone, being slightly younger or older (112). To investigate sex differences, the experiment included an equal number of males and females.

Subjects were individually housed in standard polycarbonate shoebox cages (42.5 x 20.5 x 20 cm) with hardwood chip bedding (Pine-Dry). Subjects were individually housed to prevent social and environmental enrichment effects (40; 122). Additionally,

while previous experiments maintained separate housing rooms for male and female rats (90), the present experiment utilized the same room for both sexes to more closely model potential housing environments in gender-integrated combat arms units. Cages were changed twice a week by Laboratory of Animal Medicine (LAM) staff to prevent undue stress from excess soil. Rodent chow (Harlan Teklad 4% Mouse/Rat Diet 7001) and water was made available continuously for subjects. The housing room was maintained at 23°C with 40% relative humidity on a 12-hour reverse light cycle (0500-1700 dark). Given that rats are nocturnal animals, the reverse light cycle was necessary to allow for conduct of behavioral measures during the rats' active period. Prior to data collection, rats were gentled by trained experimenters to allow for desensitization to handling and transport. To prevent potential stress effects from gentling and numbering, the procedure was conducted two days prior to behavioral testing (100). The experiment was conducted under an approved protocol by Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (MPS-14-898) and in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (National Institutes of Health Guide for Care and Use of Laboratory Animals, 2011).

Independent Variables

There were three independent variables for this study: nicotine, stress, and sex. Each variable is discussed below.

Nicotine. Nicotine bitartrate (Sigma Pharmaceuticals) was administered via osmotic mini-pump (Alzet Model 2002, Durect Corporation) in two dosages: 0 mg nic/kg/day (Saline control), 6 mg nic/kg/day. Nicotine dosages were prepared based on

average weight of subjects within each of the eight treatment conditions (see Figure 3 for sample nicotine bitartrate calculation).

The 0 mg nic/kg/day and 6 mg nic/kg/day doses represent a non-smoker and ½ to 1- pack/day smoker respectively (56; 60; 61; 65; 127). The inclusion of the 0 mg nic/kg/day and 6 mg nic/kg/day doses and not higher or lower doses were based on recent findings that suggested activity differences in rats were mostly limited to moderate doses of nicotine (90). Furthermore, the inclusion of additional nicotine doses would have required a larger sample size in each condition resulting in a logistically unwieldy experiment. Nicotine was administered via osmotic mini-pump to model continuous nicotine use in Warriors under stressful conditions. The mini-pumps were surgically implanted subcutaneously (SC) while animals were under anesthesia using a 5% isoflurane/oxygen mixture (see Figures 4 and 8 for surgery procedure details and a depiction of the implantation procedure respectively).

Stress. To model the stress from threat of death or serious injury Warriors experience during combat deployments, the experiment utilized a Warrior Stress Paradigm (WSP) for the stress manipulation. Background and rationale for the WSP is discussed further below. To isolate the stress effect from housing conditions and behavioral testing, the WSP occurred in a laboratory space that was physically separate from the housing room and behavioral rooms. Bright, florescent, overhead lighting was maintained for the duration of the WSP procedure. The WSP was conducted over a period of 14 days, wherein on each day, rats were exposed to 20-minute periods of predator stress and unpredictable environmental stress. On the first day of stress, rats were exposed to predator stress in the form of cotton balls soaked in 10mL of synthetic

fox urine (Buck Stop, Stanton, MI), which were co-located individually with each rat in a clear plastic cage (29 x 18 x 12 cm) without bedding for the entire 20-minute period. The following 13 days of stress included fox urine for the first 10 minutes, followed by an additional and varied stressor (e.g., whistle blast, coin shake, flashing lights, cage shake) at intervals for the remainder of the 20-minute period to prevent habituation to the fox urine (see Figures 5 for a detailed description of the WSP).

It should be noted that while the current WSP is conceptually similar to the model employed by Moosey (90), the current model differs in key areas. First, the current WSP extends the number of stress days utilized in Moosey (90) from 10 to 14. Second, to more closely simulate an uninterrupted period of stress (i.e., akin to a combat deployment), the current WSP model features 14 days of stress divided into 7 consecutive days with only 3 days of no stress in between (see Figure 6). This model is in contrast to the prior model, which included at least 1-2 days of no stress between each stress day (90), and more akin to an earlier model that featured one period of 7 consecutive days of stress (132). The changes made for the current model were expected to strengthen the manipulation for stress, and build on Moosey (90), which suggested that non-significant findings for a stress effect may have been secondary to the stress manipulation employed. Previous experiments utilizing the 14-day WSP or longer have reported increased biochemical markers of stress (8; 97; 114) as well as decreased depressive-like behavior in combined stress conditions (132).

Animal models of stress injury: predator stress. Predator stress has been used extensively in previous research to create behavioral phenotypes of stress-injured rats. For example, in one study, rats exposed to two 1-hour cat exposures separated by ten

days demonstrated greater increases in classically conditioned fear memory as well as generalized anxiety-like behaviors as compared to a control group (136).

Utilizing predator odor specifically is another validated method of predator stress. Trimethylthiazoline (TMT), a component of fox feces, is often used by researchers to induce stress-like reactions in rats (22; 41). Specifically, a study conducted by Endres et al. (41) reported that after exposure to TMT, rats exhibited increased fear behavior as indexed by potentiation of acoustic startle response and inhibition of appetitive behavior. Additionally, research has shown that fox urine is also a useful means to producing stress-like reactions in rats (20).

Animal model of warrior stress: warrior stress paradigm. Many of the studies that have utilized predator stress have included experiments that either exposed the rats to the stressor for only a limited period of time (20; 41; 136), or at inconsistent intervals (22). Accordingly, the WSP was developed to more closely model the consistent and prolonged exposure to stress experienced by warriors during combat deployments (133). The WSP was based on research within the Grunberg Laboratory (8; 64; 82; 90; 97; 114; 132) as well as other studies (67; 123).

The utilization of the WSP for the current study differed somewhat from more recent WSP models used within the Grunberg Laboratory (90). The current WSP involved 14 days of stress with 10 minutes exposure to fox urine followed by environmental stressors. The current paradigm was expected to produce a stronger manipulation for warrior stress because it involved a more prolonged and intense exposure to the fox urine and environmental stressors.

Sex. Given the recent consideration of including females in combat roles (18), this experiment included both males and females. With regard to sex differences within the laboratory, findings from prior experiments within the Grunberg Laboratory suggested sex differences whereby female unstressed rats receiving low and moderate doses of nicotine displayed less depressive-like behavior during open field activity (OFA) vertical activity (VA) as compared to those in the saline control condition; while this effect was not observed with male rats (90). However, these findings were reported as tentative, and the current experiment sought to further explore and possibly confirm any true sex differences.

Dependent Variables

The OFA of animals was recorded to assess for general health and activity, depressive-like, and anxiety-like behavior using measurements of horizontal activity (HA), vertical activity (VA), and center-time (CT) respectively. Descriptions of equipment, procedures, and each measurement are provided in this section.

Time. Time was included as a within-subject independent variable to allow for: observation of changes in dependent variables over time; verification of nicotine delivery to animal through body weight assessments. Behavioral measurements were collected at three time points: baseline (3 and 4 days prior to mini-pump implant and stress day 1, respectively), time 1 (between stress days 7 and 8), and time 2 (one day after the final stress day). Prior to formal data collection at baseline, a 60-minute acclimation period was conducted to allow for the animals to acclimate to the OFA environment and thereby prevent stress effects on behavioral measures (100). Please refer to Figure 2 for experimental timeline.

Equipment and Procedures. OFA is a collection of unconditioned locomotor behavior of an animal as it moves in the environment. Measurement of open field activity is a well-established procedure and has been extensively utilized in research to investigate a variety of variables, including behavioral stress responses (4; 47; 48; 65; 90; 103; 119; 132). OFA has also been used in research that has utilized the WSP (132; 90).

OFA measurements were collected using sixteen 40 x 40 x 30 cm clear Plexiglas arenas that utilize an Accuscan Superflex Sensor Version 2.2 infrared photocell system (Accuscan Instruments Incorporated, Columbus, OH). A Plexiglas lid for each arena has multiple holes to provide adequate ventilation while also preventing escape during data collection. Data is automatically gathered by the Superflex Sensors, as soon as the animal is placed in the arena, and continuously transmitted to the Accuscan Superflex Node located on the upper-rear of the OFA chamber. Data from all sixteen chambers is then transmitted to a computer via a universal serial bus (USB) connector, and processed by Accuscan Fusion Software (Version 3.4) and converted into exportable HyperText Markup Language (HTML) for further data interpretation and analysis (90; 132; 134).

Data collection was conducted during the rats' active cycle for a period of 60 minutes for each measurement time point. Once animals were individually placed in the arenas, the experimenters turned off the lights and exited the experimental room. After the 60-minute period was complete, the experimenters returned and removed each animal from the arena to be placed back in its original housing cage. The experimenter then cleaned and deodorized each arena with Clidox solution (Pharmacal, Naugatuck, CT) to ensure the proper maintenance of the arena as well as prevent undue stress from residual feces and urine.

Horizontal Activity. Horizontal activity (HA) is a measurement of the animal's general health and gross motor movement (40; 65; 66; 78; 132). The measurement is based on the number of times the animal has broken an infrared photoelectrical beam on the lower half of the OFA chamber.

Vertical activity. Vertical activity (VA) is a measure of the rats' escape-related activity (e.g., rearing), and is inversely related to depressive-like behavior whereby increased VA is indicative of less depressive-like behavior. This model is substantially based on a learned helplessness paradigm which links decreased escape activity with "depressed" animals (107) and has been successfully incorporated into animal models in previous research (4; 47; 48; 78; 90; 132). VA was computed as the number of times the animal broke an infrared photoelectric beam on the upper half of the OFA apparatus field.

Center Time. Center Time (CT) is a measurement of the animal's time spent in the center of the open field. CT is a long-established measure of anxiety-like behavior (47; 48; 65; 90; 119; 132). Given rats' natural preference for enclosed spaces (e.g., corners within the OFA chamber), CT is inversely related to anxiety-like behavior, whereby higher CT is indicative of lower anxiety-like behavior, and lower CT reflective of higher anxiety (6; 52; 77). CT was computed as a ratio of the time spent by the animal in the center of the field over the total movement time (CT/MT) in the chamber. This computation was made to account for differences in general movement activity, whereby an animal that stayed in the center and did not move around the chamber would have a higher score on CT/MT than an animal that displayed high levels of CT as a function of its general movement activity.

Experimental Timeline

Upon arrival to the Laboratory of Animal Medicine (LAM) of USUHS, animals were singly housed and randomly assigned to experimental conditions. The next day, rats were gentled and numbered by experimenters. Gentling was necessary to acclimate the rats to experimenter handling and transport; numbering was conducted by marking each tail with indelible ink to allow for proper identification of each rat. OFA acclimation was conducted on Day 5 to allow rats to desensitize to the OFA environment prior to formal measurement. Baseline (BL) OFA was conducted on Day 7. To provide a stable measurement of body weight for nicotine/saline calculations, body weights were collected on two separate occasions prior to surgical implant. After calculations were completed, on Day 9 nicotine and saline pumps were prepared based on computations derived from body weights (see Figure 3). On Day 10, rats were anesthetized with 5% oxygen-isoflorane, received analgesia via subcutaneous injection of buprenorphine (0.05 – 0.1mg/kg) and surgically implanted subcutaneously between the shoulder-blades with mini-pumps containing saline or nicotine bitartrate solution. After surgical implant, rats were re-housed in new cages (fresh bedding, water, and chow), and observed until they were able to demonstrate recovery by displaying return of right reflex and coordinated voluntary movement. Rats were also monitored three times a day until completely recovered from the surgical procedures. The stress manipulation began on Day 11 and continued through Day 17. (see Figure 6). Time 1 (T1) OFA measurements were conducted on Day 18. Stress reconvened on Day 21, and was completed on Day 27. Time 2 (T2) OFA measurements were conducted on Day 28. On Day 31, animals were anesthetized with carbon monoxide and sacrificed by decapitation. Trunk blood was

collected for future research projects. Please refer to Figure 2 for Timeline of Experiment.

Data Analytic Strategy

SPSS software (IBM, 2013) was used to conduct repeated-measures analysis of covariance (rANCOVA) on each dependent variable (HA, VA, and CT). Power analyses and resulting treatment cell sizes were based on previous studies (9; 61; 90; 132). The analyses consisted of five steps for each DV: univariate analyses of variance (ANOVAs) to examine baseline differences; omnibus rANCOVA to measure change over time (T1, T2) for each group for each DV while controlling for differences observed at BL; rANCOVAs which split for sex (male, female) to evaluate Hypotheses 2a and 2b (H2a, H2b); rANCOVAs which split for stress and sex to evaluate Hypotheses 1a – 1c (H1a, H1b, H1c); standard ANCOVAs at each time point (T1, T2) for each sex and stress sub-group (unstressed females, stressed females, unstressed males, stressed males) independently to further evaluate any significant interactions revealed by rANCOVAs. Post-hoc analyses were not conducted given that each variable did not involve more than two levels. Tests were two-tailed using α=.05. Cohen's (29) convention of small (.01 - .05), medium (.06 - .13), and large (> .14) was used for effect size (partial eta squared, η_p^2). As all of the analyses are ANCOVAs, adjusted values (adjusted means, standard errors) are reported in lieu of raw values (means, standard deviations). Data presented in text include only significant results. Graphs and all data (significant and non-significant) are presented in Appendices A and B, respectively.

CHAPTER 4: Results

Horizontal Activity (HA)

Horizontal Activity Overall Repeated-measures Analysis of Covariance

Please refer to Figures 9 and 10 for males and females, respectively. The overall rANCOVA revealed a main effect for time (F[1, 55] = 12.09, p = .001, η_p^2 = .93), such that Time 2 (T2) (12666.30, SEM = 411.33) was greater than Time 1 (T1) (11682.56, SEM = 340.60). There was a time x sex interaction (F[1, 55] = 18.68, p < .001, η_p^2 = .25), such that overall increases in activity at T2 were found for female rats but not male rats. There was a time x nicotine interaction (F[1, 55] = 5.12, p = .028, η_p^2 = .09), whereby there were differential effects over time for nicotine in male and female rats. There was also a time x stress x nicotine interaction (F[1, 55] = 10.66, p = .002, η_p^2 = .16). Finally, there was a time x sex x stress x nicotine interaction (F[1, 55] = 4.27, p = .043, η_p^2 = .07), whereby there was a 3-way time x stress x nicotine interaction in female rats, whereas in the male rats there was a 2-way time x nicotine interaction. Specifically, with regard to the male rats, the effect of nicotine occurred in converse fashion from the time x nicotine interaction observed in the female rats, whereby nicotine did not increase activity until T2, and was found for stressed and unstressed rats, while for females the time x nicotine interaction was found only in stressed female rats and not unstressed female rats.

Overall analyses at each time point separately revealed that at T2, there was a main effect for sex (F[1, 55] = 7.52, p = .008, η_p^2 = .12), whereby female rats (13894.10, SEM = 608.00) demonstrated more activity than male rats (11438.49, SEM = 608.00). There was no main effect for sex at T1. Overall analyses at each time point separately also revealed that at T2, there was a main effect for nicotine (F[1, 55] = 10.10, p = .002,

$\eta_p^2 = .16$), whereby rats that received nicotine (14026.12, SEM = 594.28) displayed more activity than rats that received saline (11306.49, SEM = 594.28). Nicotine differences were not observed at T1.

Horizontal Activity Repeated-measures Analysis of Covariance Split by Sex

Data were split by sex to examine main effects for time, stress, nicotine, and all interactions for males and females separately. Please refer to Figures 9 and 10 for male and female rats, respectively. For male rats there was no main effect for time. For male rats, there was a time x nicotine interaction ($F[1, 27] = 6.28$, $p = .02$, $\eta_p^2 = .19$) such that rats that received nicotine had greater HA than rats that received saline at T2 while this difference was not observed at T1. To further evaluate this finding, standard ANCOVAs at each time point for each sub-group of male rats were conducted (see below). There was no main effect for stress.

For female rats there was a main effect for time ($F[1, 27] = 19.85$, $p < .001$, $\eta_p^2 = .42$) such that T2 (14400.13, SEM = 678.10) was greater than T1 (12333.28, SEM = 542.66). For female rats, there was also a time x stress x nicotine interaction ($F[1, 27] = 13.50$, $p = .001$, $\eta_p^2 = .33$), whereby there was a significant time x nicotine 2-way interaction for stressed female rats which was not observed in unstressed female rats. This finding will be further elucidated in the section below for stress and sex split. There were no main effects for stress or nicotine.

Horizontal Activity Repeated-measures Analysis of Covariance, Split by Sex and Stress

For unstressed males, there were no significant main effects or interaction effects. Additionally, ANCOVAs at T1 and T2 did not reveal any main effects for nicotine.

For stressed males, there was no main effect for time. There was a main effect for nicotine ($F[1, 13] = 5.82$, $p = .03$, $\eta_p^2 = .31$), such that rats that received nicotine displayed higher HA than rats that received saline. Follow-on ANCOVAs revealed that at T2 there was a main effect for nicotine ($F[1, 13] = 7.67$, $p = .016$, $\eta_p^2 = .37$), such that rats that received nicotine (12815.08, SEM = 838.53) displayed greater HA than rats that received saline (9531.92, SEM = 838.53); no such effect was found at T1.

For unstressed female rats, there was a main effect for time ($F[1, 13] = 8.49$, $p = .01$, $\eta_p^2 = .40$) such that activity at T2 (14226.88, SEM = 878.40) was greater than T1 (12775.36, SEM = 706.95). There was no main effect for nicotine. Standard ANCOVAs conducted at T1 and T2 did not reveal significant effects for nicotine.

For stressed female rats, there was a main effect for time ($F[1, 13) = 14.31$, $p = .002$, $\eta_p^2 = .52$), such that activity at T2 (14573.38, SEM = 1066.97) was greater than T1 (11891.19, SEM = 835.24). There was no main effect for nicotine. There was a significant time x nicotine interaction ($F[1, 13] = 6.03$, $p = .029$, $\eta_p^2 = .32$), such that rats that received nicotine had higher HA than rats that received saline at T1, while this difference was not observed at T2 (Figure 10). Follow on ANCOVA at T1 revealed a main effect for nicotine ($F[1, 13] = 6.02$, $p = .029$, $\eta_p^2 = .32$) whereby rats that received nicotine (14002.76, SEM = 1199.40) displayed more HA than rats (9779.61, SEM = 1199.40) that received saline. The ANCOVA at T2 in turn did not reveal a significant main effect for nicotine.

Summary of Horizontal Activity

There was a significant time x sex interaction, whereby for female rats there was an increase in HA over time, which was not observed in male rats. There were significant

time x nicotine interactions which differed depending on sex and stress. For female rats, the time x nicotine interaction occurred in only those that were stressed, whereby in this condition, rats that received nicotine displayed more HA than those that received saline at T1, while no difference between groups were observed at T2. The time x nicotine x stress interaction for female rats is explained by the fact that this two-way interaction between time and nicotine was not found in unstressed female rats. On the other hand for male rats, the time x nicotine interaction occurred in converse fashion, whereby the effect of nicotine did not increase HA until T2. For male rats, the significant differences for nicotine at T2 were found in those that were stressed as with female rats at T1; however, the differences between stressed and unstressed male rats compared at T1 and T2 did not reach significance to become a third interaction term, hence the 4-way interaction described earlier.

Vertical Activity (VA)

Vertical Activity Overall Repeated-measures Analysis of Covariance

Please refer to Figures 11 and 12 for male and female rats, respectively. The overall rANCOVA revealed a main effect for time ($F[1, 55] = 4.90$, $p = .031$, $\eta_p^2 = .082$), such that activity at T2 (1785.09, SEM = 68.10) was greater than T1 (1474.64, SEM = 57.21). There was a significant main effect for sex ($F[1, 55] = 4.17$, $p = .046$, $\eta_p^2 = .07$), whereby male rats (1743.22, SEM = 76.80) overall demonstrated more vertical activity than female rats (1516.52, SEM = 76.80). Upon examining overall differences at each time point separately, there was a main effect for sex at T1 ($F[1, 55] = 8.68$, $p = .005$, $\eta_p^2 = .14$), such that males (1651.05, SEM = 82.81) demonstrated more activity than females

(1298.23, SEM = 82.81); no differences were observed at T2. There were no significant interactions.

Vertical Activity Repeated-measures Analysis of Covariance, Split by Sex

Data were split by sex to examine main effects for time, stress, nicotine, and all interactions for males and females separately. Please refer to Figures 11 and 12 for male and female rats, respectively. For male rats, there were no main effects for time, stress, or nicotine; and no significant interactions. For female rats, there was a main effect for time $(F[1, 27] = 6.68, p = .015, \eta_p^2 = .20)$, such that T2 (1800.25, SEM = 105.76) was greater than T1 (1384.03, SEM = 73.56). For female rats, there was also a time x stress x nicotine interaction $(F[1, 27] = 6.52, p = .017, \eta_p^2 = .20)$. There were no main effects for stress or nicotine.

Vertical Activity Repeated-measures Analysis of Covariance, Split by Sex and Stress

For unstressed males, there were no main effects for time or nicotine; and no significant interactions. For stressed males, there were no main effects for time or nicotine; and no significant interactions. Standard ANCOVAs at T1 did not reveal any effects for nicotine for all sub-groups. The ANCOVA at T2 for stressed males revealed a main effect for nicotine $(F[1, 13] = 5.70, p = .033, \eta_p^2 = .31)$, whereby rats that received nicotine (2033.46, SEM = 149.74) displayed higher levels of VA than those that received saline (1520.17, SEM = 149.74). For unstressed females, there were no main effects for time or nicotine; and no significant interactions. For stressed females, there were no main effects for time or nicotine; and no significant interactions.

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Summary of Vertical Activity

Pairwise comparisons revealed that at T2, stressed male rats demonstrated more VA when receiving nicotine versus saline. For females there was a main effect for time, whereby VA increased from T1 to T2. While this effect was not observed for males, the interaction of time x sex, while approaching significance, did not reach it.

Center Time (CT)

<u>Center Time Overall Repeated-measures Analysis of Covariance</u>

Please refer to Figures 13 and 14 for male and female rats, respectively. As indicated earlier, CT was computed as a ratio of the time spent by the animal in the center of the field over the total movement time (CT/MT) in the chamber. The overall rANCOVA did not reveal any main effects or interaction effects.

<u>Center Time Repeated-measures Analysis of Covariance, Split by Sex</u>

Data were split by sex to examine main effects for time, stress, nicotine, and all interactions for males and females separately. Please refer to Figures 13 and 14 for male and female rats, respectively. For male rats, there were no main effects for time, stress, or nicotine; and no significant interactions. For female rats, there was a main effect for time $(F[1, 27] = 5.02, p = .034, \eta_p^2 = .16)$, such that activity at T2 (13.31, SEM = 1.82) was higher than T1 (12.36, SEM = 1.93). There were no main effects for stress or nicotine; and no significant interactions.

<u>Center Time Repeated-measures Analysis of Covariance, Split by Sex and Stress</u>

For unstressed male rats, there were no main effects for time or nicotine; and no significant interactions. For stressed male rats, there were no main effects for time, nicotine, and no significant interactions; albeit the time x nicotine interaction was

approaching significance (F[1, 13] = 4.12, p = .063, η_p^2 = .24). For unstressed female rats,

there were no main effects for time or nicotine; and no significant interactions. For

stressed female rats, there were no main effects for time or nicotine; and no significant

interactions. Standard ANCOVAs at T1 and T2 did not reveal any effects for nicotine for

any sub-groups.

Summary of Center Time

For female rats, CT increased from T1 to T2. Nicotine did not have any

significant effects.

CHAPTER 5: Evaluation of Hypotheses

The purpose of the present experiment was to determine the effects of nicotine, stress, and sex on depressive-like and anxiety-like behavior in rats. Each hypothesis is evaluated in this section. Specific aims and hypotheses are re-stated for reference.

Specific Aim 1: To examine the effects of nicotine on depressive-like and anxiety-like behavior under stressful and non-stressful conditions.

Hypothesis 1a: The hypothesis that stressed rats would exhibit more depressive-like and anxiety-like behaviors as compared to unstressed rats as measured respectively by OFA vertical activity (VA) and center time (CT) **was not confirmed**. There were no significant main effects for stress on either depressive-like or anxiety-like behaviors in male or female rats.

Hypothesis 1b: The hypothesis that nicotine would attenuate depressive-like and anxiety-like behavior for stressed and non-stressed rats **was not confirmed**. There were no significant main effects for nicotine on either depressive-like or anxiety-like behaviors in male or female rats.

Hypothesis 1c: The hypothesis that nicotine would be a protective factor against stress as indicated by lower levels of depressive-like and anxiety-like behaviors in stressed animals administered nicotine as compared to stressed animals administered saline **was partially confirmed**. Stressed male rats that received nicotine at T2 displayed more VA (less depressive-like behavior) than stressed male rats that received saline. However, this finding is associated with limitations, and will be discussed in the following section. Additionally, no other effects of nicotine in stressed groups were observed for either male or female rats for depressive-like or anxiety-like behavior.

Specific Aim 2: To determine whether there are sex differences with regard to the effects of stress and nicotine on depressive-like and anxiety-like behavior.

     Hypothesis 2a: The hypothesis that stressed male rats would display decreased levels of depressive-like and anxiety-like behaviors as compared to stressed female rats **was not confirmed**. The effect of stress did not differ significantly depending on sex for depressive-like and anxiety-like behavior.

     Hypothesis 2b: The hypothesis that nicotine would produce greater attenuation of depressive-like and anxiety-like behaviors for female rats as compared to male rats **was not confirmed**. The effect of nicotine did not differ significantly depending on sex for depressive-like and anxiety-like behavior.

CHAPTER 6: Discussion

Given the relationship between nicotine use, stress, and modulation of mood (51; 115; 19; 84; 58; 76), this experiment was designed to experimentally investigate the effects of nicotine, stress, and sex on behavioral indices of anxiety and depression in animals. Previous animal research has implicated the following: utilization of the Warrior Stress Paradigm (WSP) produces increases in depressive-like and anxiety-like behavior in male and female rats (9; 132; 134); females are more sensitive to the effects of predatory and environmental stress as indicated by increased levels of depressive-like and anxiety-like behaviors (9; 132; 134); nicotine attenuates depressive-like behavior for unstressed female rats (90). Contrary to hypotheses, our present experiment for the most part did not confirm significant effects for nicotine or stress on anxiety-like or depressive-like behavior.

Depressive-like behavior was assessed by measuring vertical activity (VA) within an open field apparatus. Our experimental results indicated that at T2, there was increased vertical activity (less depressive-like behavior) for stressed male rats receiving nicotine as compared to stressed male rats receiving saline. These results would seem to suggest that nicotine was providing a protective effect against stress for male rats. However, upon consideration of the increased horizontal activity (HA) at T2 of stressed male rats receiving nicotine as compared to stressed male rats receiving saline, it would appear that differences in vertical activity in this group are due to the effect of nicotine to increase activity in general. Overall, nicotine increases activity (HA and VA) for stressed males at T2. The finding that nicotine increased horizontal activity will be discussed further below in the context of other research that has investigated nicotine's role in

enhancing motor activity. After considering these findings together, it is difficult to confirm any general effects of nicotine, or any protective effects for nicotine against stress on depressive-like behavior from this experiment.

From earlier studies, it is also difficult to establish any clear effects of nicotine on behavioral indices of depression in comparably aged Sprague-Dawley (SD) rats (47; 46; 90). While Faraday, Elliott, and Grunberg (47), reported VA as being unaffected by 12mg/kg/day nicotine for male and female SD rats; in contrast, Faraday, O'Donoghue, and Grunberg (46) reported increased VA from 6 mg/kg/day for male and female SD rats. It is possible that the differences in nicotine dosages between these two studies underlie the different findings for VA. To further explore, a more recent experiment utilizing a similar approach to the current experiment (90) reported an effect of 6 mg/kg/day nicotine to increase VA for unstressed female SD rats. However, this finding was garnered from a less conservative statistical analysis, and no such effects were found for stressed rats. In summary, the literature is unclear with regard to nicotine's effect on depressive-like behavior.

As with depressive-like behavior, there were no clear effects for either nicotine or stress on anxiety-like behavior, which was assessed by measuring center time in OFA in the present experiment. With regard to the effects of nicotine on anxiety-like behavior, the literature is uncertain. Faraday, Elliott, and Grunberg (47) found that CT was unaffected by a higher dose of nicotine (12 mg/kg/day) for adult (60 days old) male SD rats, while increasing CT for adult female SD rats. The findings for female rats in this experiment were not explained by general increases in activity, as horizontal activity was unaffected for these rats. Conversely, Elliott, Faraday, Phillips, and Grunberg (39) found

increased anxiety-like behavior in male and female adult (60 days) SD rats; while these effects differed from general activity as measured by OFA. However, it is worth noting that this experiment differed in two key areas: it utilized a different measure of anxiety-like behavior, the elevated plus maze; it administered lower doses of nicotine (.5 and 1 mg/kg/day). In summary, with regard to nicotine's effect on anxiety-like behavior, due to substantial differences between experimental procedures and measurements (e.g., differences in routes of administration, dosages, behavioral measures), it is difficult to draw stable conclusions from the available literature as well as draw meaningful comparisons with the current experiment.

Additionally, and seemingly contrary to what would be suggested by previous research (8; 9; 82; 97; 114; 132; 134), this experiment failed to reproduce increases in depressive-like and anxiety-like behavior from WSP. Without a clear effect for stress, it is natural to postulate that nicotine could not be protective against a stress effect that was not apparent, which would explain the findings described above.

Previous research within the Grunberg Lab has indicated that predator stress in the form of fox urine has been capable of producing increased levels of biochemical markers of stress (corticosterone) in rats (8; 9; 82; 97; 114). Berger (8) demonstrated increased serum corticosterone in the offspring of mothers who had been stressed while they were in utero. Long (2010) described an increase in fecal corticosterone during a stress period which combined WSP and sleep disruption. Starosciak (114) reported a main effect for stress on serum corticosterone, as well as causing disruption to drinking and eating behavior. Similar to Long (82), it is important to note here that in Perry (97), a pair of experiments that utilized a similar 14-day WSP, the increase in corticosterone was

observed in a combined stress condition only (sleep deprivation and predator stress), with no effects from the predator stressor alone.

With regard to behavioral indices of depression (vertical activity, forced swim test) and anxiety (center time, elevated plus maze), previous research has mostly failed to confirm any significant detrimental effects from the WSP *in isolation* (8; 82; 97; 90; 114; 132). Berger (8) discovered an interesting interaction between stress and social isolation whereby male rats that were stressed and isolated displayed less CT (more anxiety-like behavior) as compared to other groups; albeit no main effects for stress alone on CT, EPM, or FST. Starosciak (114) utilized a WSP that occurred over a period of 17 consecutive days (longest to date), and found no main effects for stress on VA, CT, or FST. Starosciak (114) reported that stressed animals displayed intermediate depressive-like scores on the FST, and that there was a significant interaction between ethanol and stress, whereby ethanol reduced the effect of stress on FST; however, the main effect of stress on depressive-like behavior in FST was not significant. Long (82) did not find any effects for stress on CT or FST. It is pertinent to note here that Long (82) utilized a combined WSP and sleep manipulation, whereby the WSP occurred over a period of non-consecutive days, which is in contrast to most other models of WSP, in which the stress days occur over a period of consecutive days.

It is true that Yarnell (132), discovered decreases in vertical activity and center-time for female rats, however, these changes were found in the combined condition (blast and psychological stress) only. As a final note, while the age ranges for Yarnell (132) and Moosey (90) were more comparable to the current experiment's age range of 51-55 (54 days and 52 days respectively), Perry (97) utilized a much younger age range during the

WSP (22-25 days). In other research, a mild restraint stressor also failed to yield a significant effect for stress on VA (46). In summary, the literature is generally unsupportive with regard to stand-alone WSP or mild stressors in having an impact on depressive-like and anxiety-like behavior in rats. Our findings from the present experiment seem to coincide with this notion.

While not included as a specific aim in this experiment, the findings for nicotine's effect on horizontal activity are worth noting. While previous research has strongly suggested that similar age-ranged SD rats are not as sensitive to the effect of moderate doses of nicotine on horizontal activity as compared to Long-Evans rats (7; 13; 46; 83); more recent research (90) has indicated that nicotine in moderate doses (3 mg/kg/day, 6 mg/kg/day) has an activating effect in SD rats.

Our present findings are more in line with Moosey (90), and given stronger resemblance between these two studies, would seem to provide replication. Moosey (90) found that nicotine at moderate doses (3 mg/kg/day, and 6 mg/kg/day) had an initial activating effect for female rats as indicated by increased HA at T1, which then decreased at subsequent time points. This pattern was more profound with unstressed females versus stressed females. In our present experiment, a similar pattern was discovered for female rats, whereby there were increases in HA at T1 that were not observed at T2; albeit for stressed rats and not unstressed rats as was found with Moosey (90). Another finding from the present experiment, which also coincides with Moosey (90) was that for males, nicotine increased activity for mainly those that were stressed, although this effect did not reach significance. However, in contrast to Moosey (90) the increase in HA for male rats was not seen until T2. In summary, the findings for HA, while mitigating

against prior research utilizing substantially different procedures and variables (7; 13; 46; 83), are closely in line with more recent research that bears substantial conceptual, technical, and procedural resemblance (90). While nicotine had activating effects on male and female rats, female rats were more sensitive to these activating effects as compared to male rats.

Limitations

Independent variables. There are some limitations with the experiment's independent variables. With regard to nicotine, it is possible that inclusion of higher or lower doses may have helped establish some of the differential findings according to dose discussed earlier. In particular, research has found effects on VA from 6 mg/kg/day nicotine (46; 90) but not from 12 mg/kg/day nicotine (47). Additionally, with regard to CT, research has indicated increased behavior for females and not males at higher doses (47); and increased behavior for males and females from lower doses (39). As the present experiment included only the 0 mg/kg/day and 6 mg/kg/day conditions and did not find any effects of nicotine on CT, it is yet undetermined whether or not changes in CT scores are a reflection of dose-dependent responses, sensitivity of the CT measure, or both.

With regard to stress, it seems clear that the WSP in isolation (regardless of WSP model), has limited effect on behavioral indices of depression and anxiety (8; 82; 97; 90; 114; 132). It is possible, that given the reliance of WSP on singular usage of fox urine and different environmental stressors, it is not strong enough as a stand-alone manipulation for stress when utilizing pure behavioral measures. Researchers seem to have found consensus that either the WSP is not strong enough; behavioral measures are not sensitive enough; or both (8; 90; 114).

With regard to housing, the present experiment utilized the same room for both male and female rats, with separate sections of the room designated for each sex respectively. Prior experiments have been conducted with males and females housed in separate rooms (90). While the experiment sought to more closely model a gender-integrated Warrior housing condition, it is possible that housing animals in separate rooms may moderate the effect of other independent variables on behavior. Furthermore, animals were individually housed in this experiment. As reported previously, crowded housing conditions differentially affect bio-behavioral responses in male and female rats (15).

Dependent variables. There are some limitations with the experiment's dependent variables. With regard to depressive-like behavior, the experiment singularly utilized vertical activity within OFA. Other studies have utilized different measures of depressive-like behavior, including the forced-swim test (FST) (99). With regard to the FST, research has indicated increased depressive-like behavior as a result of chronic variable stress (89; 96). Similarly, with regard to anxiety-like behavior, the experiment solely relied on measurements of center time within OFA. As discussed earlier, other experiments have found effects utilizing elevated plus maze (38; 44).

Future Directions

Independent variables. To coincide with the aforementioned limitations, future research may build on the independent variables included in this experiment. While considering nicotine, future research may be encouraged to include lower and higher doses of nicotine to better establish their effects on behavioral markers of anxiety and depression. In consideration of stress, it may be of pertinence for future experiments to

enhance the WSP by including other stressors which have been found to have effects on behavioral measures of depression and anxiety. The current experiment's WSP relied solely on predator and environmental stress. Given previous research findings (97), sleep deprivation may be an interesting stressor to consider including. Furthermore, there are other independent variables that were not included at all in this experiment that bear note. One such variable, rat strain, may be an interesting inclusion given the differential findings reported in the literature (46; 97). Age range may be another interesting inclusion given differential findings regarding adolescent versus adult rats (47; 97). By investigating different age ranges of rats, future experiments may add to the literature regarding younger versus older Warriors. Finally, including a housing variable with regard to the room (both sexes in the same room, each sex in separate rooms) or the cage (individually housed, socially housed) may help distinguish any differential effects that result from how males and females are separated or from the number of animals housed per cage respectively. Relatedly, while this experiment based its power analyses and treatment cell sizes on prior experiments (9; 61; 90; 132), it is possible that smaller effect sizes could be detected with a greater sample size. Additionally, with consideration towards including additional variables mentioned above, the overall sample size of the experiment would need to be increased to attain adequate power to detect significant findings.

Dependent variables. Future research may build on the dependent variables included in this experiment. In terms of measuring depressive-like behavior, the forced swim test is another well-established measure (99) that may possibly be used in addition to VA. In the same vein, future experiments may wish to incorporate other measures of

anxiety-like behavior, such as elevated plus maze, which has been successfully used in prior research (38). Furthermore, given that there are over 7,000 chemicals produced from tobacco smoke (e.g., formaldehyde, arsenic, ammonia), future research may wish to explore these chemicals, their interactions with nicotine, and effects on biology and behavior (118). Finally, while the current experiment solely utilized behavioral indices of depression and anxiety, the role of nicotine and stress in male and female rats may be better illuminated by measuring biological markers of depression and anxiety.

CHAPTER 7: Summary

The present experiment set out to investigate the effects of nicotine, stress, and sex on behavioral markers of depression and anxiety in rats. The experiment did not reveal any significant effects for nicotine, stress, or stress on measures of depressive-like or anxiety-like behavior. As discussed earlier, this finding may be explained by limitations with regard to the strength of the stress manipulation with the current WSP; inadequate measures for depression and anxiety; or both. However, with regard to general activity as measured by HA, the experiment closely replicated recent research within the Grunberg Lab. In terms of HA, the experiment found that the activating effect for nicotine occurs sooner for females as compared to males; and that this activating effect appeared to mainly occur for rats that were stressed. The fact that the activating effect of nicotine mainly occurred in animals that were stressed, may provide implications for the Warrior population. Upon consideration of the Yerkes-Dodson performance and arousal curve (135), the activation effect of nicotine may be functional for the performance of Warriors during periods of stress (over-arousal), such as deployment. Additionally, this activation effect may also be explained in the context of the fight-or-flight response (21), whereby nicotine provides Warriors increased motor response during periods of stress and danger.

CHAPTER 8: Conclusion

There were no clear effects for nicotine, stress, or sex on depressive-like or anxiety-like behavior in rats. Accordingly, hypotheses with regard to depressive-like and anxiety-like behavior are unconfirmed. Nicotine, however, did increase general activity for both sexes, with females displaying more activity sooner than males, and for mainly rats that were stressed. In lieu of answering methodological limitations addressed earlier, from the present findings, it seems apt to postulate that nicotine may serve to generally increase the activity-levels of Warriors, possibly more so for Warriors that have experienced stress. This finding may be a functional consequence of the fight-or-flight stress response (21), whereby nicotine increases the ability of the Warrior to actively respond to stressful and dangerous situations.

APPENDIX A – FIGURES
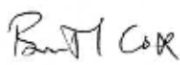
UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

April 2, 2014

MEMORANDUM FOR DR. NEIL GRUNBERG, DEPARTMENT OF MEDICAL AND
CLINICAL PSYCHOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services
University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee
(IACUC) via Designated Member Review on April 2, 2014:

Title of Application: "Behavioral investigations of nicotine and caffeine in rats (Rattus
norvegicus)"

USUHS Protocol Number: MPS-14-898

Expiration Date: April 1, 2017

Supporting Grant(s) Number: E072194414

Name of Principal Investigator: Dr. Neil Grunberg

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory
Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is
A3448-01. The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review
must be completed by the anniversary date of the protocol. If work is to be continued past the
expiration date, a triennial review must be completed prior to the expiration date in order for
work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work
may be done without an approved protocol. Although the IACUC may send reminders, it is the
investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in
advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ. Amanda Christy to schedule
a pre-protocol planning meeting (295-3708). This meeting must occur to ensure animal numbers
are loaded in the CART system and LAM resources are available to meet your needs.

Brian M. Cox, Ph.D.
Chair, Institutional Animal
Care and Use Committee

cc: Office of Research

Figure 1. IACUC Approval of Protocol
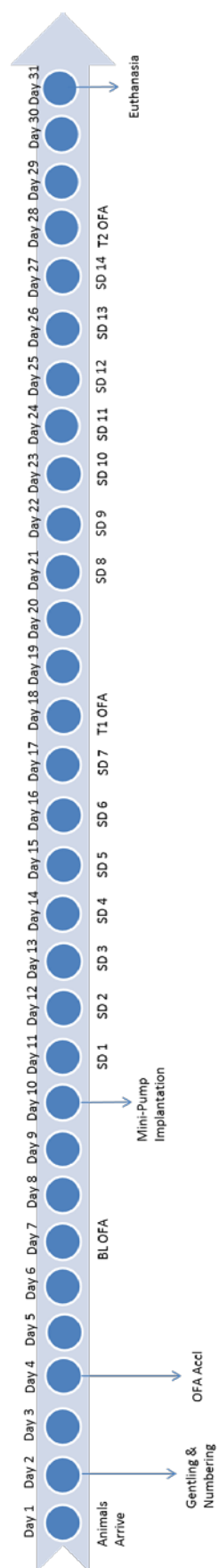
Figure 2. Timeline of Experiment

OSMOTIC PUMP SPECIFICATIONS:
The following information can be found in the package insert:
Model # 2002
Lot # 10269-12
Mean *in vitro* pump rate = 0.42 µl/hr
Mean *in vitro* pump duration = 22 day(s)
Mean pump fill volume = 247

Mean pump fill volume = (*in vitro* pump rate) x (24 hr/1 day) x (*in vitro* pump duration)
= (.**42** µl/hr) x (24 hr/1 day) x (22 day(s))
= **221.76** µl

In vivo pump rate conversion factor (CF) = 0.95*
(*Note: CF is constant for all ALZET osmotic pumps)
Daily *in vivo* pump rate = (CF) x (*in vitro* pump rate) x (24 hr/1 day)
= (0.95) x (.**42** µl/hr) x (24 hr/1 day)
= **9.576** µl/day

Pump operation duration = (pump fill volume)/(daily *in vivo* pump rate)
= (**247** µl) / (**9.576** µl/day)
= **25.79** day(s)*
(*Note: Duration is calculated to confirm sufficient number of days for the experiment)

NICOTINE BASE DOSAGE SOLUTION (males):
Dose concentration = (dosage choice) / [(daily *in vivo* pump rate) x (1 ml/1,000 µl)]
= (**6** mg/kg/ day) / [(**9.576** µl/day) x (1 ml/1,000 µl)]
= **626.57** mg Nic. base/kg/ml

Nicotine Base concentration = (mean animal body weight) x (dose concentration)
= (.**277** kg) x (**626.57** mg Nic. base/kg/ml)
= **173.56** mg Nic. Base./ml
Nicotine base to Nicotine Bitartrate conversion factor (CF) = 2.850*
(*Note: CF is constant for all Nic. Bit. conversions)
- CF= Formula weight of nicotine Bitartrate/ Molecular Weight Nicotine
Nicotine Bitartrate conversion = (Nic. Base. concentration) x (2.850)
= (**173.56** mg Nic. Base./ml) x (2.850)
= **494.65** mg Nic. Bit./ml

TOTAL SALINE VOLUME:
Total Saline solution volume = (pump fill volume) x (1 ml/1,000 µl) x (number of animals)
= (**247** µl) x (1 ml/1,000 µl) x (20 rats)
= **4.94** ml (minimum total volume)*
(*Note: You may increase the total Nicotine solution volume in order to ensure adequate solution volume for the experiment)

TOTAL AMOUNT OF NICOTINE BITARTRATE:
Nicotine Bitartrate = (total Saline volume) x (Nicotine concentration)
= (**4.94** ml) x (**494.65** mg Nic. Bit./ml)
= **2.4435** g Nic. Bit.

ACTUAL PROPORTION CALCULATIONS:
Actual amount of Nicotine Bitartrate measured = 4.66 g Nic. Bit.
Actual amount of Nicotine Bitartrate needed = **2.4435** g Nic.
Conversion factor (CF) = (A) / (B)
= (4.66 g Nic. Bit.) / (2.4435 g Nic. Bit.)
= 1.907
ACTUAL SALINE VOLUME:
Actual Saline volume = (total Saline volume) x (CF)
= (**4.94** ml) x (1.907)
= 9.42 ml Saline to add

NICOTINE BASE DOSAGE SOLUTION (females):
Dose concentration = (dosage choice) / [(daily *in vivo* pump rate) x (1 ml/1,000 µl)]
= (**6** mg/kg/ day) / [(**9.576** µl/day) x (1 ml/1,000 µl)]
= **626.57** mg Nic. base/kg/ml

Nicotine Base concentration = (mean animal body weight) x (dose concentration)
= (.**199** kg) x (**626.57** mg Nic. base/kg/ml)
= **124.69** mg Nic. Bit./ml

Nicotine base to Nicotine Bitartrate conversion factor (CF) = 2.850*
(*Note: CF is constant for all Nic. Bit. Conversions)
- CF= Formula weight of nicotine Bitartrate/ Molecular Weight Nicotine
Nicotine Bitartrate conversion = (Nic. Base concentration) x (2.850)
= (**124.69** mg Nic. Base /ml) x (2.850)
= **355.37** mg Nic. Bit./ml

TOTAL SALINE VOLUME:
Total Saline volume = (pump fill volume) x (1 ml/1,000 µl) x (number of animals)
= (**247** µl) x (1 ml/1,000 µl) x (16 rats)
= **3.95** ml (minimum total volume)*
(*Note: You may increase the total Nicotine solution volume in order to ensure adequate solution volume for the experiment)

TOTAL AMOUNT OF NICOTINE BITARTRATE:
Nicotine Bitartrate = (total Saline volume) x (Nicotine concentration)
= (**3.95** ml) x (**355.37** mg Nic. Bit./ml)
= **1.40** g Nic. Bit.

ACTUAL PROPORTION CALCULATIONS:
Actual amount of Nicotine Bitartrate measured = 3.77 g Nic. Bit.
Actual amount of Nicotine Bitartrate needed = **1.40** g Nic. Bit.
Conversion factor (CF) = (D) / (E)
= (3.77 g Nic. Bit.) / (1.40 g Nic. Bit.)
= 2.692
ACTUAL SALINE VOLUME:
Actual Saline volume = (total Saline volume) x (CF)
= (**3.95** ml) x (2.692)
= 10.64 ml Saline to add

Figure 3. Sample Nicotine Bitartrate Calculation (Cohort 2)

- Note: pump preparation and labeling should be complete at this point
- Reserve Surgery prep room, isofluorane, knock-out box, 2 x isofluorane masks, O2, heating pads for cages, scissors (at least 2 x 6 inch short/curved nosed to tent; and 2 x long/straight nose to cut), biohazard bags, scavengers, trash bin, and LAM personnel support by completing form and also notifying LAM vet personnel in person
- Establish accountability (go/no roster) and identify roles/responsibilities for day of surgery. Disseminate this a week prior to date. Specific roles may include
    - Supervision/General assistance
    - Anesthesia & Rat shaver supervision
    - Surgeons x 2
    - Recorder
    - Animal transport and post-operative care
    - Animal transport, clean-up, general assistance
- At least day before ensure everything set up in surgery room
- Ensure all equipment has been inspected, cleaned, organized, and consolidated in the wet lab (at least 2 days prior to sacrifice). Items may include (but see specific list and diagram, separate document):
    - 3-4 rolls of paper towels
    - Scissors
    - Stapler, staples, stapler removal tool (2 sets)
- Conduct mock run-through morning of surgery with all required personnel. Ensure room is set up the way you need it. Practice oral communication procedures (e.g., calling out rat numbers, and pump # confirmation)
    - Ensure Vet techs are provided education on how to shave (this is very important as it will impact the surgery cut)
- Post-operative: use betadyne ointment after rats transported back to housing room

Figure 4. Surgery Procedure Guidance Form

Predatory Stressor	Procedure
Fox Urine	Ensure each cotton ball has fully absorbed 10mL of synthetic fox urine before placing the urine-soaked ball into the stress cages for 20 minutes on Day 1 and for 10 minutes on all subsequent days. On Day 1, the animals do not receive any additional environmental stressors. For Days 2-14, at the end of 10 minutes, remove cotton ball from the container and begin additional environmental stressors.
Environmental Stressor	**Procedure**
Main lights flash	Flash overhead lights six times using light switch randomly at designated times during the latter 10 minutes
Whistle	Blow whistle for 3-4 seconds randomly at designated times during the latter 10 minutes
Cage shaking	Pick each cage up sequentially and shake in a figure 8 motion 4-5 times at 3 separate intervals within 10 minutes.
Coins in metal container	Shake 5 times at designated times during the latter 10 minutes. The coin container should be held by the side to ensure proper sound.

Figure 5. Warrior Stress Paradigm (WSP) Description and Procedure

Stress Day	Predator Stress	Unpredictable Event
1	**Fox Urine** (20 min)	**None**
2	**Fox Urine** (10 min)	**Whistle** at 12, 15 & 19 min
3	**Fox Urine** (10 min)	**Coin Shake** at 11, 14, & 17 min
4	**Fox Urine** (10 min)	**Flashing Lights** at 13, 16, & 19 min
5	**Fox Urine** (10 min)	**Cage Shake** at 12, 15, & 18 min
6	**Fox Urine** (10 min)	**Flashing Lights** at 12, 16, & 19 min
7	**Fox Urine** (10 min)	**Whistle** at 11, 13, 16 & 18 min
8	**Fox Urine** (10 min)	**Coin Shake** at 12, 16, & 19 min
9	**Fox Urine** (10 min)	**Flashing Lights** at 11, 15, 19 min
10	**Fox Urine** (10 min)	**Cage Shake** at 11, 14, & 17 min
11	**Fox Urine** (10 min)	**Coin Shake** at 13, 16, & 19 min
12	**Fox Urine** (10 min)	**Whistle** at 12, 14, 17 min
13	**Fox Urine** (10 min)	**Flashing Lights** at 11, 14, 18 min
14	**Fox Urine** (10 min)	**Cage Shake** at 12, 15, & 18 min

Figure 6. WSP Schedule
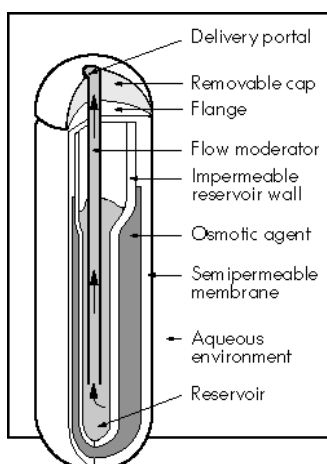
Figure 7. Cross-section illustration of the ALZET Model 2002 Minipump

Image by ALZET (2012)

Figure 8. Subcutaneous (SC) location of Minipump Implant in the Rat.

Image by ALZET (2012)
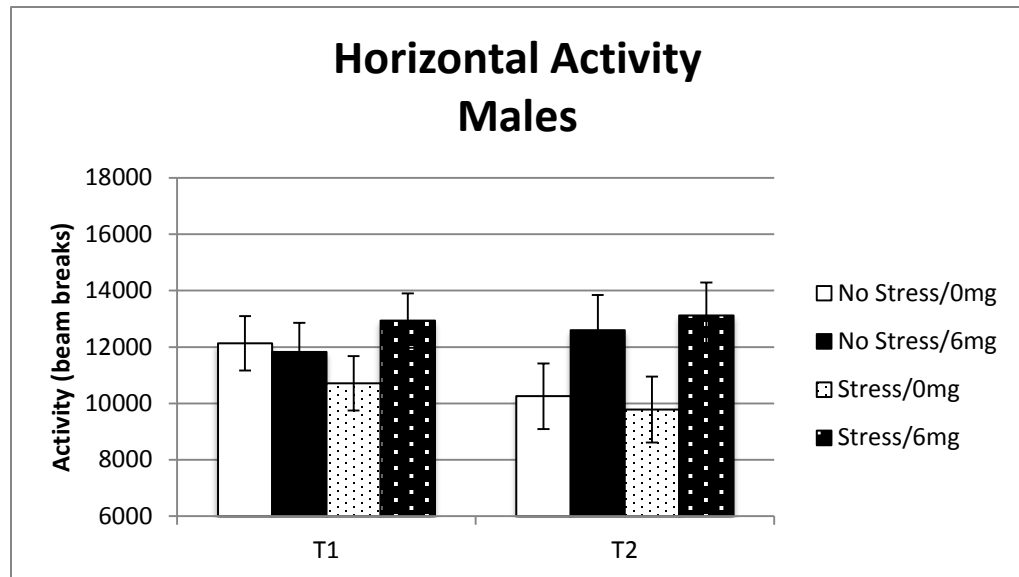
Figure 9. Horizontal Activity, Males, Co-varied for Baseline
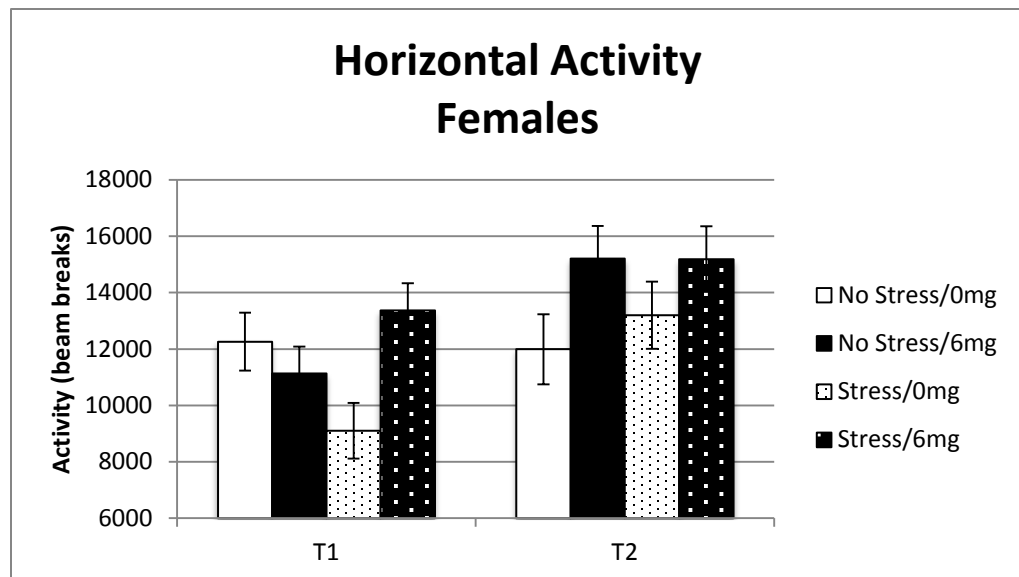
Figure 10. Horizontal Activity, Females, Co-varied for Baseline
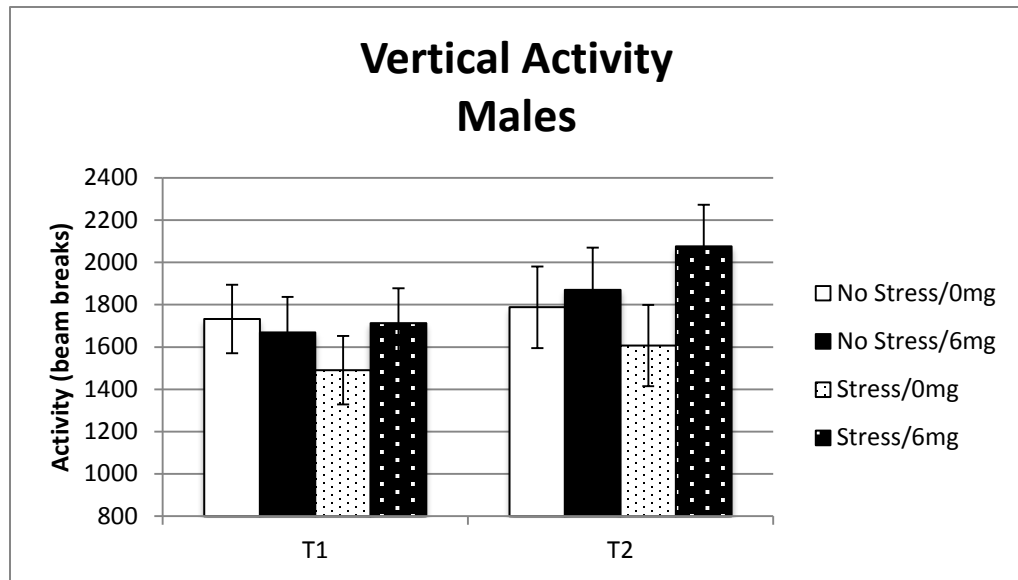
Figure 11. Vertical Activity, Males, Co-varied for Baseline
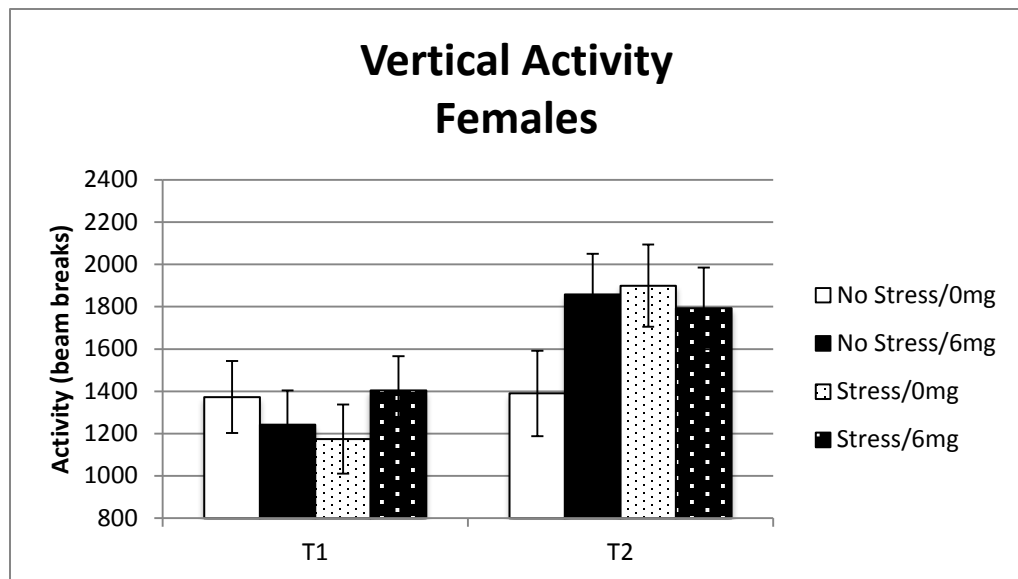
Figure 12. Vertical Activity, Females, Co-varied for Baseline
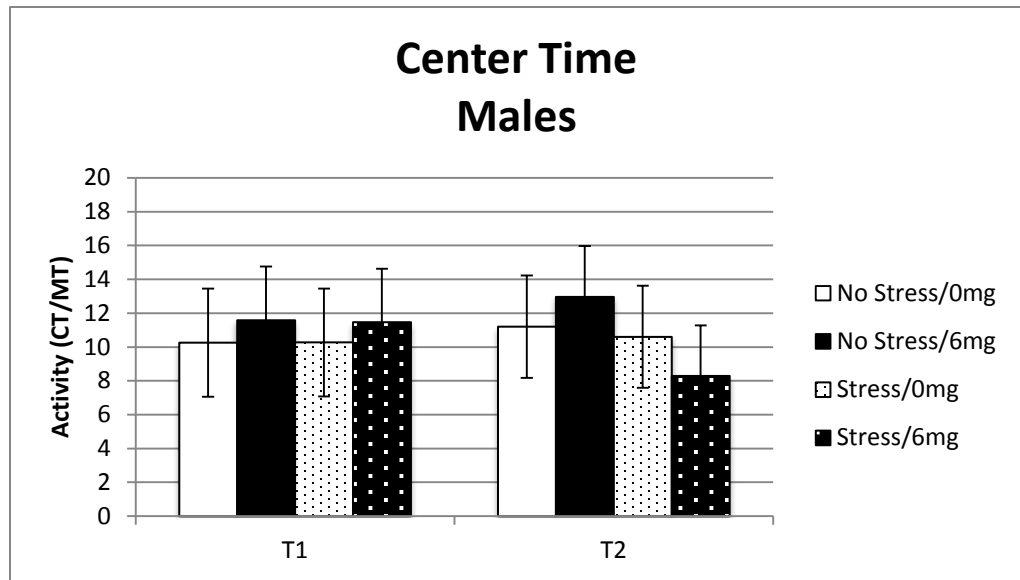
Figure 13. Center Time, Males, Co-varied for Baseline
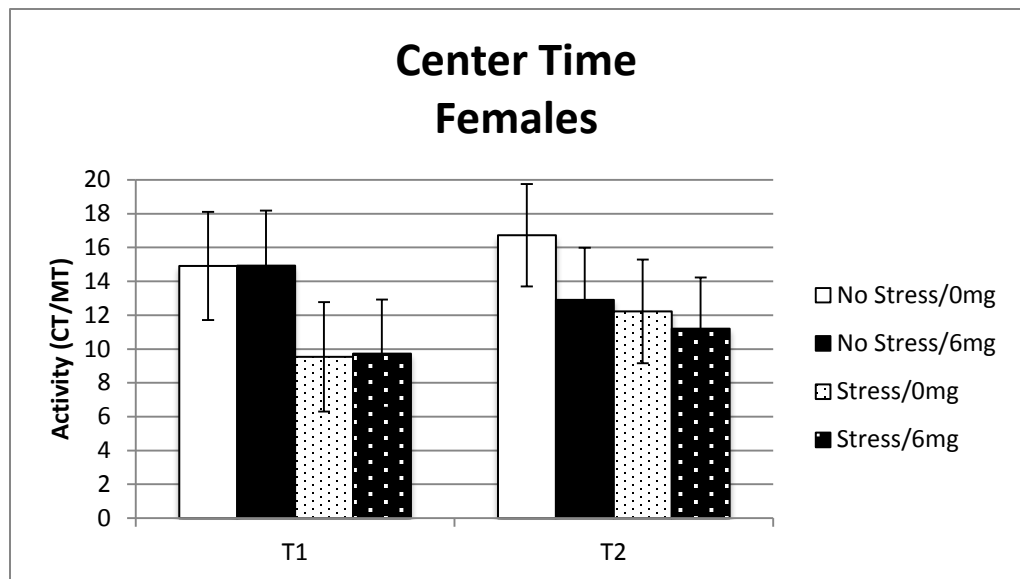
Figure 14. Center Time, Females, Co-varied for Baseline
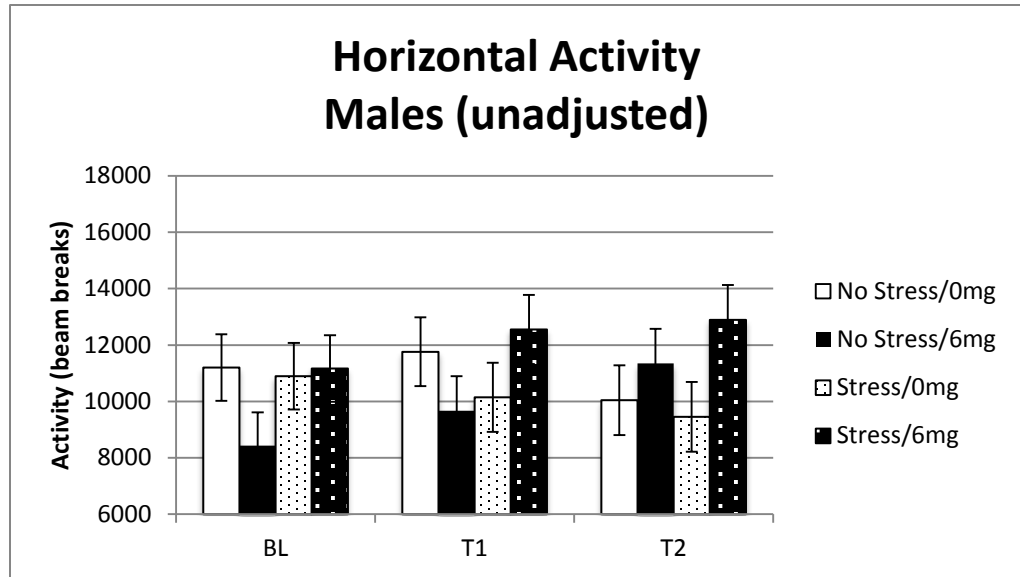
Figure 15. Horizontal Activity, Males, Unadjusted for Baseline
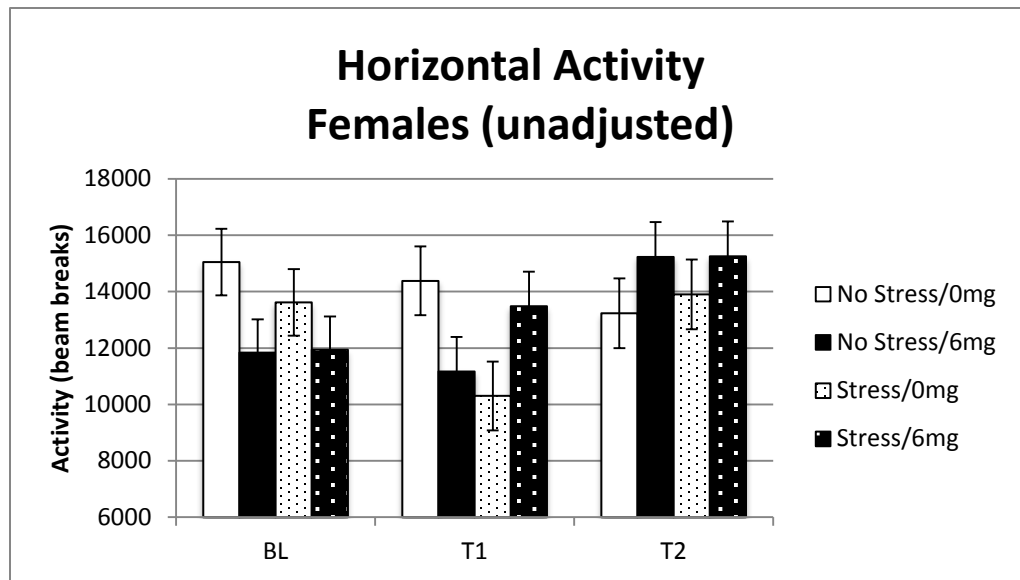
Figure 16. Horizontal Activity, Females, Unadjusted for Baseline
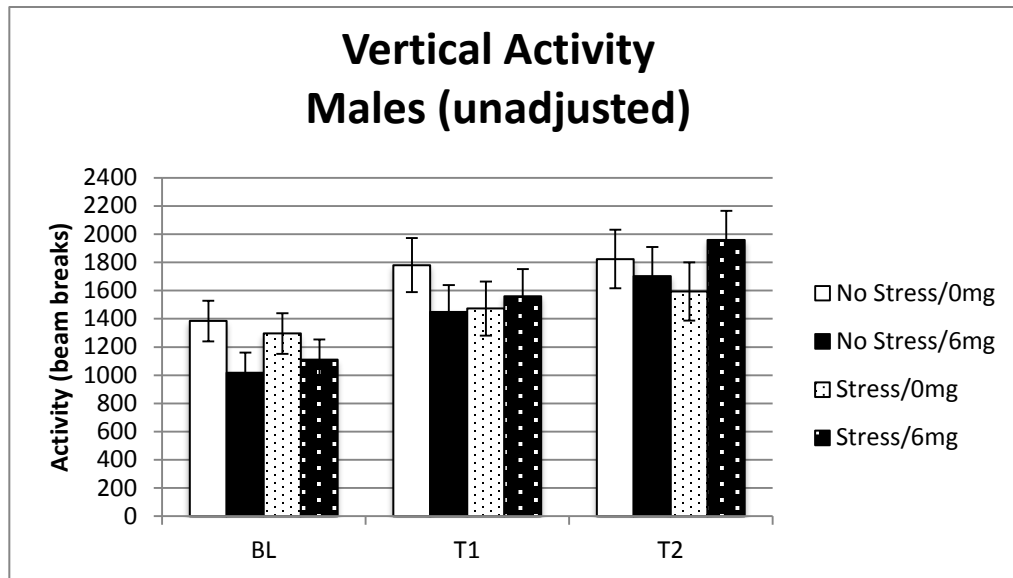
Figure 17. Vertical Activity, Males, Unadjusted for Baseline
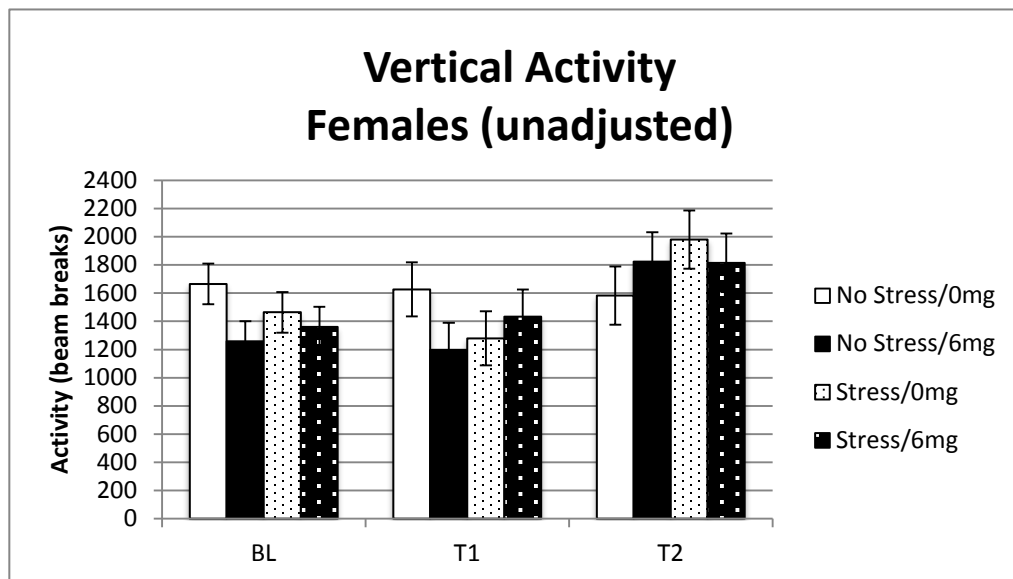
Figure 18. Vertical Activity, Females, Unadjusted for Baseline
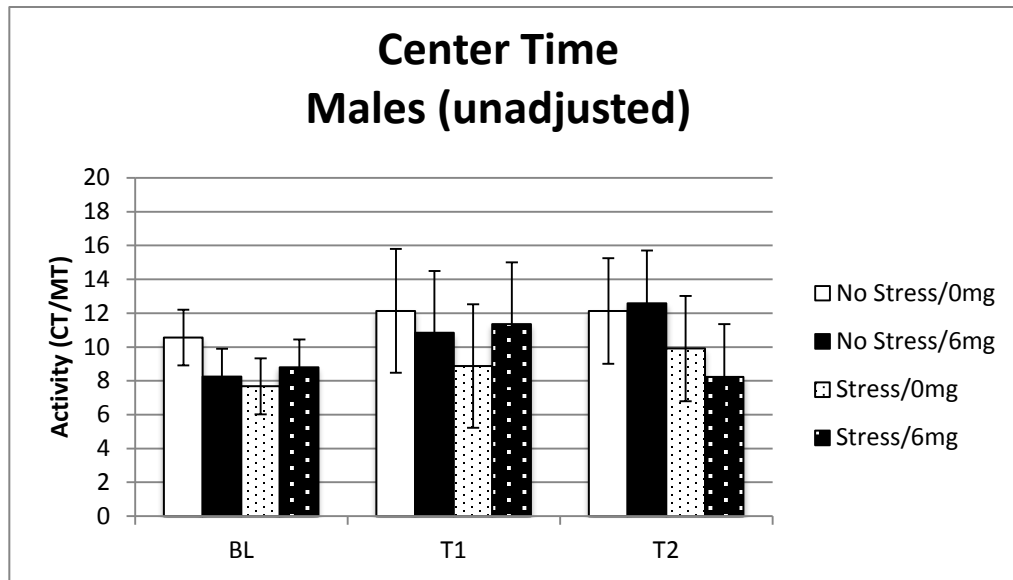
Figure 19. Center Time, Males, Unadjusted for Baseline
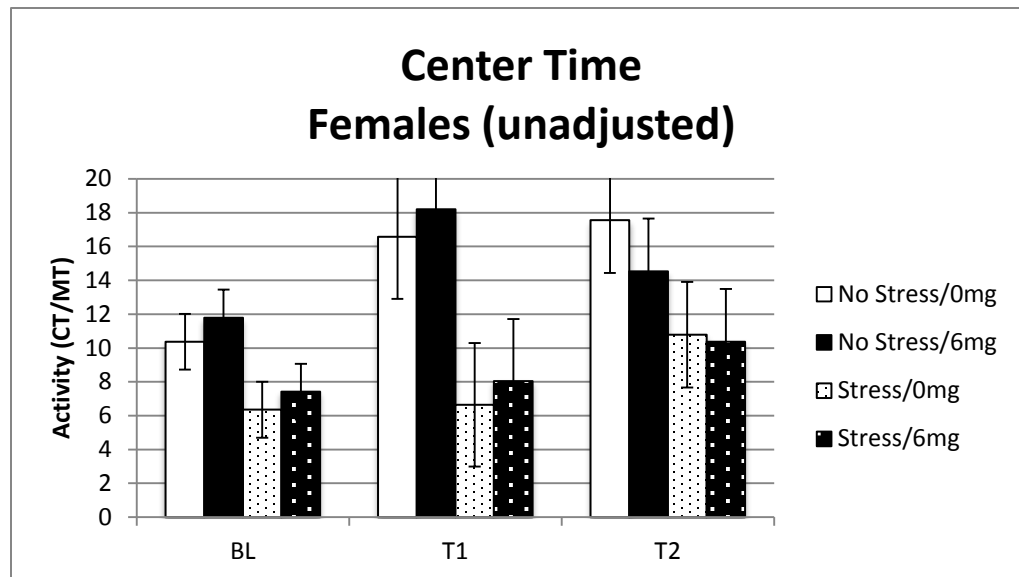
Figure 20. Center Time, Females, Unadjusted for Baseline
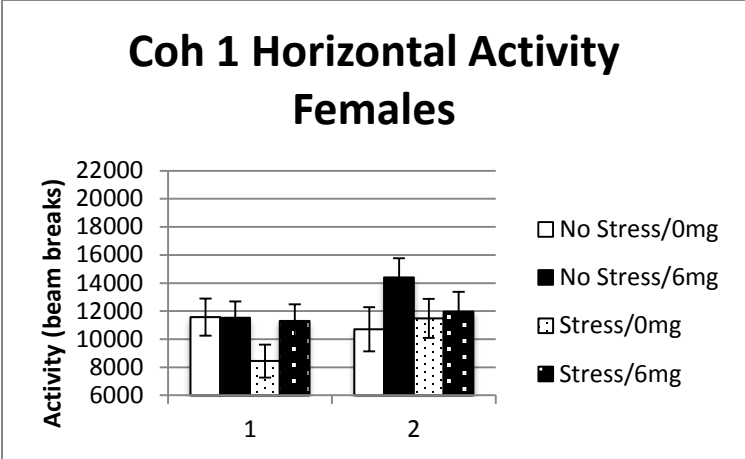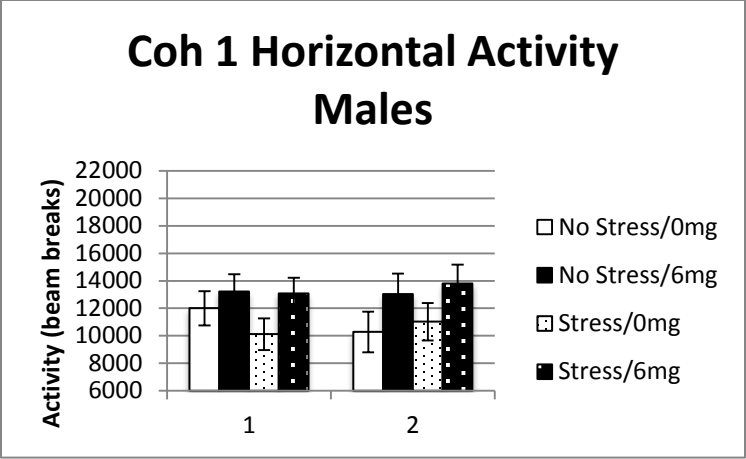
Figure 21. Cohort 1 Horizontal Activity, Males and Females (Co-varied for Baseline)
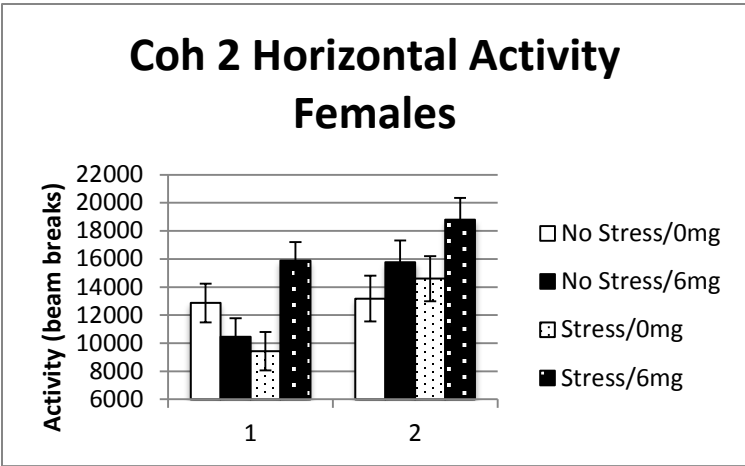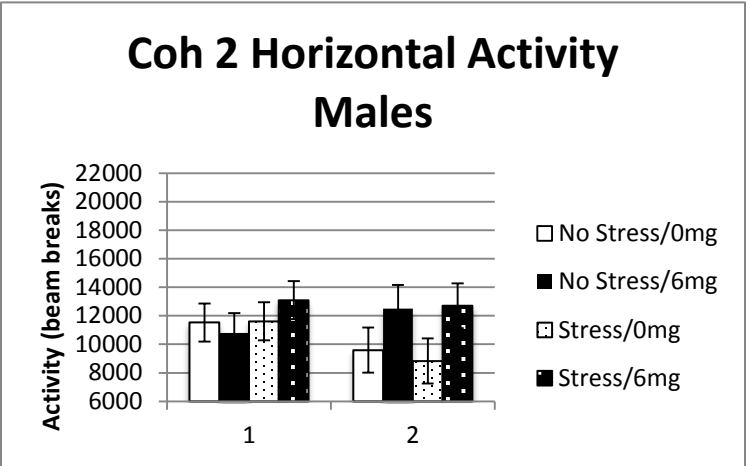
Figure 22. Cohort 2 Horizontal Activity, Males and Females (Co-varied for Baseline)
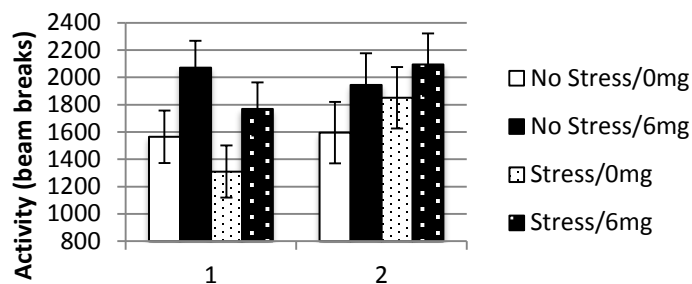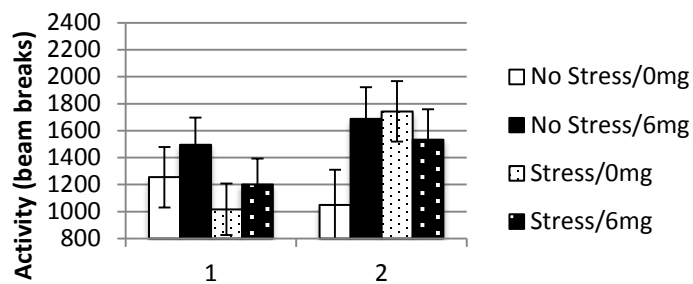
Figure 23. Cohort 1 Vertical Activity, Males and Females (Co-varied for Baseline)
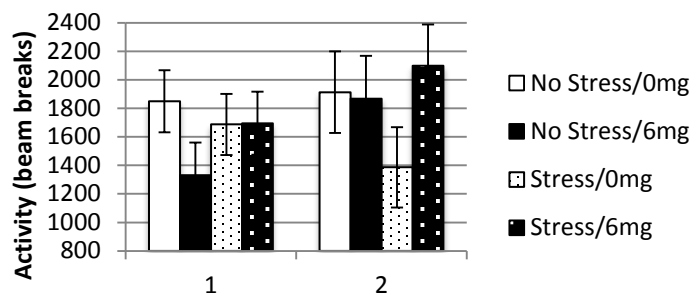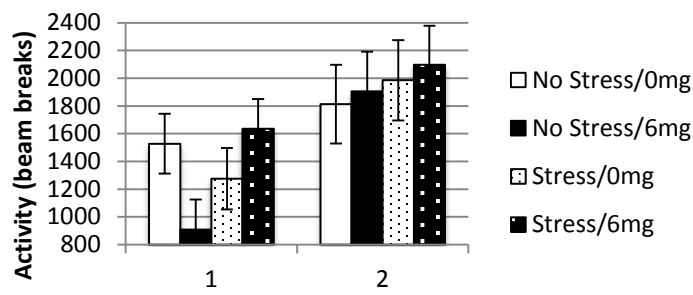
Figure 24. Cohort 2 Vertical Activity, Males and Females (Co-varied for Baseline)

Figure 25. Cohort 1 Center Time, Males and Females (Co-varied for Baseline)

Figure 26. Cohort 2 Center Time, Males and Females (Co-varied for Baseline)

APPENDIX B – TABLES

Table 1. Treatment cell breakdown

Subject Breakdown (N=64) (Cell Size = 8)		
Sex	Male	32
	Female	32
Stress	No Stress	32
	Stress	32
Nicotine	0 mg/kg	32
	6 mg/kg	32

Table 2. Overall rANCOVA of Horizontal Activity

Overall rANCOVA of Horizontal Activity Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	36579088.344	1	36579088.344	12.092	.001	.180	.927
Time * BLHA	22854376.536	1	22854376.536	7.555	.008	.121	.770
Time * Sex	56505903.684	1	56505903.684	18.679	.000	.254	.989
Time * Stress	3028923.157	1	3028923.157	1.001	.321	.018	.166
Time * Nic	15481150.570	1	15481150.570	5.118	.028	.085	.604
Time * Sex * Stress	1488083.619	1	1488083.619	.492	.486	.009	.106
Time * Sex * Nic	1426753.021	1	1426753.021	.472	.495	.009	.104
Time * Stress * Nic	32245689.662	1	32245689.662	10.660	.002	.162	.894
Time * Sex * Stress * Nic	12919820.172	1	12919820.172	4.271	.043	.072	.528
Error(Time)	166376576.277	55	3025028.660				

Overall rANCOVA of Horizontal Activity Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	318208797.35	1	318208797.35	20.899	.000	.275	.994
BLHA	327491432.78	1	327491432.78	21.509	.000	.281	.995
Sex	27505391.190	1	27505391.190	1.806	.184	.032	.262
Stress	254.238	1	254.238	.000	.997	.000	.050
Nic	117044171.83	1	117044171.83	7.687	.008	.123	.778
Sex * Stress	138527.234	1	138527.234	.009	.924	.000	.051
Sex * Nic	266152.713	1	266152.713	.017	.895	.000	.052
Stress * Nic	28682505.812	1	28682505.812	1.884	.175	.033	.271
Sex * Stress * Nic	217738.746	1	217738.746	.014	.905	.000	.052
Error	837422851.78	55	15225870.032				

Table 3. rANCOVA of Horizontal Activity, Males

rANCOVA of Horizontal Activity, Male Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	395405.466	1	395405.466	.142	.709	.005	.065
Time * BLHA	593453.951	1	593453.951	.214	.648	.008	.073
Time * Stress	19183.680	1	19183.680	.007	.934	.000	.051
Time * Nic	17453642.418	1	17453642.418	6.284	.019	.189	.676
Time * Stress * Nic	4411955.290	1	4411955.290	1.589	.218	.056	.229
Error(Time)	74987527.799	27	2777315.844				

rANCOVA of Horizontal Activity, Male Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Intercept	153315398.050	1	153315398.050	15.614	.001	.366	.968
BLHA	159770258.994	1	159770258.994	16.272	.000	.376	.973
Stress	86368.923	1	86368.923	.009	.926	.000	.051
Nic	56257622.143	1	56257622.143	5.730	.024	.175	.636
Stress * Nic	11280985.784	1	11280985.784	1.149	.293	.041	.179
Error	265107734.756	27	9818804.991				

Table 4. rANCOVA of Horizontal Activity, Females

rANCOVA of Horizontal Activity, Female Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	58436911.921	1	58436911.921	19.848	.000	.424	.990
Time * BLHA	34157171.702	1	34157171.702	11.602	.002	.301	.907
Time * Stress	3426741.414	1	3426741.414	1.164	.290	.041	.180
Time * Nic	1156722.353	1	1156722.353	.393	.536	.014	.093
Time * Stress * Nic	39716510.749	1	39716510.749	13.490	.001	.333	.943
Error(Time)	79492799.360	27	2944177.754				

rANCOVA of Horizontal Activity, Female Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Intercept	165708118.55	1	165708118.551	7.819	.009	.225	.769
BLHA	167816587.06	1	167816587.065	7.918	.009	.227	.774
Stress	64661.177	1	64661.177	.003	.956	.000	.050
Nic	59619205.672	1	59619205.672	2.813	.105	.094	.366
Stress * Nic	17440483.262	1	17440483.262	.823	.372	.030	.141
Error	572219703.74	27	21193322.361				

Table 5. rANCOVA of Horizontal Activity, Male No Stress

rANCOVA of Horizontal Activity, Male No Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	3303458.791	1	3303458.791	.960	.345	.069	.149
Time * BLHA	3507650.182	1	3507650.182	1.020	.331	.073	.155
Time * Nic	8703131.245	1	8703131.245	2.530	.136	.163	.314
Error(Time)	44723357.693	13	3440258.284				

rANCOVA of Horizontal Activity, Male No Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Intercept	63686423.272	1	63686423.272	6.611	.023	.337	.662
BLHA	21371090.798	1	21371090.798	2.218	.160	.146	.282
Nic	2688972.846	1	2688972.846	.279	.606	.021	.078
Error	125240945.077	13	9633918.852				

Table 6. rANCOVA of Horizontal Activity, Male Stress

rANCOVA of Horizontal Activity, Male Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	92537.586	1	92537.586	.044	.837	.003	.054
Time * BLHA	23964.702	1	23964.702	.011	.917	.001	.051
Time * Nic	2110499.887	1	2110499.887	1.004	.335	.072	.153
Error(Time)	27326009.173	13	2102000.706				

rANCOVA of Horizontal Activity, Male Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Intercept	85581800.231	1	85581800.231	8.123	.014	.385	.750
BLHA	141300035.589	1	141300035.589	13.411	.003	.508	.922
Nic	61263690.780	1	61263690.780	5.815	.031	.309	.607
Error	136965922.286	13	10535840.176				

Table 7. rANCOVA of Horizontal Activity, Female No Stress

rANCOVA of Horizontal Activity, Female No Stress Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	34838532.944	1	34838532.944	8.492	.012	.395	.768
Time * BLHA	26248671.895	1	26248671.895	6.398	.025	.330	.648
Time * Nic	16955868.501	1	16955868.501	4.133	.063	.241	.469
Error(Time)	53335535.980	13	4102733.537				

rANCOVA of Horizontal Activity, Female No Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Intercept	51846712.121	1	51846712.121	3.193	.097	.197	.380
BLHA	97634251.533	1	97634251.533	6.012	.029	.316	.621
Nic	9650889.503	1	9650889.503	.594	.455	.044	.110
Error	211108898.842	13	16239146.065				

Table 8. rANCOVA of Horizontal Activity, Female Stress

rANCOVA of Horizontal Activity, Female Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	25849895.803	1	25849895.803	14.310	.002	.524	.937
Time * BLHA	10582338.242	1	10582338.242	5.858	.031	.311	.610
Time * Nic	10894004.281	1	10894004.281	6.031	.029	.317	.623
Error(Time)	23483424.945	13	1806417.303				

rANCOVA of Horizontal Activity, Female Stress, Between Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Intercept	118412397.330	1	118412397.330	4.295	.059	.248	.484
BLHA	72874807.632	1	72874807.632	2.643	.128	.169	.325
Nic	68722997.114	1	68722997.114	2.493	.138	.161	.310
Error	358418332.805	13	27570640.985				

Table 9. ANOVA of Horizontal Activity, Male No Stress, Baseline

ANCOVA of Horizontal Activity, Male No Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	30567076.563	1	30567076.563	6.230	.026	.308	.642
Intercept	1542309620.063	1	1542309620.063	314.336	.000	.957	1.000
Nic	30567076.563	1	30567076.563	6.230	.026	.308	.642
Error	68691966.375	14	4906569.027				
Total	1641568663.000	16					
Corrected Total	99259042.938	15					

Table 10. ANOVA of Horizontal Activity, Male Stress, Baseline

ANCOVA of Horizontal Activity, Male Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	307193.063	1	307193.063	.019	.892	.001	.052
Intercept	1945625935.563	1	1945625935.563	121.092	.000	.896	1.000
Nic	307193.063	1	307193.063	.019	.892	.001	.052
Error	224942292.375	14	16067306.598				
Total	2170875421.000	16					
Corrected Total	225249485.438	15					

Table 11. ANOVA of Horizontal Activity, Female No Stress, Baseline

ANCOVA of Horizontal Activity, Female No Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	41299902.250	1	41299902.250	3.917	.068	.219	.454
Intercept	2889008750.250	1	2889008750.250	274.018	.000	.951	1.000
Nic	41299902.250	1	41299902.250	3.917	.068	.219	.454
Error	147603813.500	14	10543129.536				
Total	3077912466.000	16					
Corrected Total	188903715.750	15					

Table 12. ANOVA of Horizontal Activity, Female Stress, Baseline

ANCOVA of Horizontal Activity, Female Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	11336689.000	1	11336689.000	.869	.367	.058	.140
Intercept	2612129881.000	1	2612129881.000	200.240	.000	.935	1.000
Nic	11336689.000	1	11336689.000	.869	.367	.058	.140
Error	182630038.000	14	13045002.714				
Total	2806096608.000	16					
Corrected Total	193966727.000	15					

Table 13. ANCOVA of Horizontal Activity, Male No Stress, T1

ANCOVA of Horizontal Activity, Male No Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	38551043.314	2	19275521.657	4.345	.036	.401	.646
Intercept	18990270.239	1	18990270.239	4.281	.059	.248	.482
BLHA	21097448.252	1	21097448.252	4.756	.048	.268	.523
Nic	858440.693	1	858440.693	.194	.667	.015	.069
Error	57667709.623	13	4435977.663				
Total	1933905611.000	16					
Corrected Total	96218752.938	15					

Table 14. ANCOVA of Horizontal Activity, Male Stress, T1

ANCOVA of Horizontal Activity, Male Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	92025322.504	2	46012661.252	6.558	.011	.502	.828
Intercept	45651335.430	1	45651335.430	6.506	.024	.334	.655
BLHA	68821833.504	1	68821833.504	9.808	.008	.430	.825
Nic	20316210.915	1	20316210.915	2.895	.113	.182	.351
Error	91216421.246	13	7016647.788				
Total	2243176126.000	16					
Corrected Total	183241743.750	15					

Table 15. ANCOVA of Horizontal Activity, Female No Stress, T1

ANCOVA of Horizontal Activity, Female No Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	153794505.754	2	76897252.877	9.617	.003	.597	.945
Intercept	842465.024	1	842465.024	.105	.751	.008	.060
BLHA	112565264.754	1	112565264.754	14.077	.002	.520	.934
Nic	511224.636	1	511224.636	.064	.804	.005	.056
Error	103952901.996	13	7996377.077				
Total	2869110710.000	16					
Corrected Total	257747407.750	15					

Table 16. ANCOVA of Horizontal Activity, Female Stress, T1

ANCOVA of Horizontal Activity, Female Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	110066151.911	2	55033075.955	4.930	.026	.431	.704
Intercept	16805310.533	1	16805310.533	1.506	.242	.104	.206
BLHA	69498806.348	1	69498806.348	6.226	.027	.324	.636
Nic	67170310.301	1	67170310.301	6.018	.029	.316	.622
Error	145106776.527	13	11162059.733				
Total	2517578371.000	16					
Corrected Total	255172928.438	15					

Table 17. ANCOVA of Horizontal Activity, Male No Stress, T2

ANCOVA of Horizontal Activity, Male No Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	10563410.791	2	5281705.395	.611	.557	.086	.131
Intercept	47999611.825	1	47999611.825	5.557	.035	.299	.588
BLHA	3781292.728	1	3781292.728	.438	.520	.033	.094
Nic	10533663.398	1	10533663.398	1.219	.289	.086	.176
Error	112296593.147	13	8638199.473				
Total	1951769377.000	16					
Corrected Total	122860003.938	15					

Table 18. ANCOVA of Horizontal Activity, Male Stress, T2

ANCOVA of Horizontal Activity, Male Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	119850327.788	2	59925163.894	10.661	.002	.621	.964
Intercept	40023002.387	1	40023002.387	7.120	.019	.354	.694
BLHA	72502166.788	1	72502166.788	12.898	.003	.498	.912
Nic	43057979.753	1	43057979.753	7.660	.016	.371	.725
Error	73075510.212	13	5621193.093				
Total	2190479474.000	16					
Corrected Total	192925838.000	15					

Table 19. ANCOVA of Horizontal Activity, Female No Stress, T2

ANCOVA of Horizontal Activity, Female No Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	27313658.924	2	13656829.462	1.106	.360	.145	.203
Intercept	85842780.041	1	85842780.041	6.953	.021	.348	.684
BLHA	11317658.674	1	11317658.674	.917	.356	.066	.144
Nic	26095533.369	1	26095533.369	2.114	.170	.140	.271
Error	160491532.826	13	12345502.525				
Total	3426268748.000	16					
Corrected Total	187805191.750	15					

Table 20. ANCOVA of Horizontal Activity, Female Stress, T2

ANCOVA of Horizontal Activity, Female Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Corrected Model	21275364.526	2	10637682.263	.584	.572	.082	.127
Intercept	127456982.600	1	127456982.600	6.997	.020	.350	.687
BLHA	13958339.526	1	13958339.526	.766	.397	.056	.128
Nic	12446691.094	1	12446691.094	.683	.423	.050	.120
Error	236794981.224	13	18214998.556				
Total	3656202488.000	16					
Corrected Total	258070345.750	15					

Table 21. Overall rANCOVA of Vertical Activity

Overall rANCOVA of Vertical Activity Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	714986.436	1	714986.43	4.903	.031	.082	.585
Time * BLVA	139418.179	1	139418.17	.956	.332	.017	.161
Time * Sex	464804.676	1	464804.67	3.188	.080	.055	.419
Time * Stress	248185.500	1	248185.50	1.702	.197	.030	.249
Time * Nic	191416.450	1	191416.45	1.313	.257	.023	.203
Time * Sex * Stress	33395.198	1	33395.198	.229	.634	.004	.076
Time * Sex * Nic	8639.320	1	8639.320	.059	.809	.001	.057
Time * Stress * Nic	340334.568	1	340334.56	2.334	.132	.041	.323
Time * Sex * Stress * Nic	535447.325	1	535447.32	3.672	.061	.063	.469
Error(Time)	8019703.25	55	145812.78				

Overall rANCOVA of Vertical Activity Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	5981699.658	1	5981699.658	16.600	.000	.232	.979
BLVA	7697721.845	1	7697721.845	21.362	.000	.280	.995
Sex	1501823.388	1	1501823.388	4.168	.046	.070	.518
Stress	27403.670	1	27403.670	.076	.784	.001	.058
Nic	611895.999	1	611895.999	1.698	.198	.030	.249
Sex * Stress	167362.667	1	167362.667	.464	.498	.008	.103
Sex * Nic	31297.273	1	31297.273	.087	.769	.002	.060
Stress * Nic	102902.554	1	102902.554	.286	.595	.005	.082
Sex * Stress * Nic	393335.458	1	393335.458	1.092	.301	.019	.177
Error	19818620.84	55	360338.561				

Table 22. rANCOVA of Vertical Activity, Males

rANCOVA of Vertical Activity, Male Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	81043.781	1	81043.781	.463	.502	.017	.101
Time * BLVA	2118.971	1	2118.971	.012	.913	.000	.051
Time * Stress	49557.898	1	49557.898	.283	.599	.010	.081
Time * Nic	195594.565	1	195594.565	1.118	.300	.040	.175
Time * Stress * Nic	5451.448	1	5451.448	.031	.861	.001	.053
Error(Time)	4725046.404	27	175001.719				

rANCOVA of Vertical Activity, Male Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	2955189.200	1	2955189.200	9.512	.005	.261	.844
BLVA	5310540.635	1	5310540.635	17.093	.000	.388	.978
Stress	30048.185	1	30048.185	.097	.758	.004	.060
Nic	649473.253	1	649473.253	2.090	.160	.072	.286
Stress * Nic	384422.704	1	384422.704	1.237	.276	.044	.189
Error	8388538.740	27	310686.620				

Table 23. rANCOVA of Vertical Activity, Females

rANCOVA of Vertical Activity, Female Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	794147.480	1	794147.480	6.678	.015	.198	.702
Time * BLVA	221063.103	1	221063.103	1.859	.184	.064	.260
Time * Stress	219070.147	1	219070.147	1.842	.186	.064	.258
Time * Nic	35110.831	1	35110.831	.295	.591	.011	.082
Time*Stress *Nic	775537.132	1	775537.132	6.521	.017	.195	.692
Error(Time)	3210892.959	27	118921.961				

rANCOVA of Vertical Activity, Female Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	3115225.067	1	3115225.067	7.558	.011	.219	.755
BLVA	2688692.971	1	2688692.971	6.523	.017	.195	.692
Stress	145636.714	1	145636.714	.353	.557	.013	.088
Nic	103082.425	1	103082.425	.250	.621	.009	.077
Stress * Nic	19185.229	1	19185.229	.047	.831	.002	.055
Error	11128570.341	27	412169.272				

Table 24. rANCOVA of Vertical Activity, Male No Stress

rANCOVA of Vertical Activity, Male No Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	74769.392	1	74769.392	.439	.519	.033	.094
Time * BLVA	24436.908	1	24436.908	.143	.711	.011	.064
Time * Nic	38758.582	1	38758.582	.227	.641	.017	.073
Error(Time)	2215608.279	13	170431.406				

rANCOVA of Vertical Activity, Male No Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1419320.275	1	1419320.275	3.790	.074	.226	.438
BLVA	2868435.326	1	2868435.326	7.659	.016	.371	.725
Nic	31645.051	1	31645.051	.084	.776	.006	.058
Error	4868655.362	13	374511.951				

Table 25. rANCOVA of Vertical Activity, Male Stress

rANCOVA of Vertical Activity, Male Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	16655.904	1	16655.904	.087	.772	.007	.059
Time * BLVA	8324.607	1	8324.607	.044	.838	.003	.054
Time * Nic	164081.635	1	164081.635	.861	.370	.062	.138
Error(Time)	2478795.580	13	190676.583				

rANCOVA of Vertical Activity, Male Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1537223.409	1	1537223.409	5.691	.033	.304	.598
BLVA	2450309.209	1	2450309.209	9.071	.010	.411	.795
Nic	1005364.075	1	1005364.075	3.722	.076	.223	.431
Error	3511679.478	13	270129.191				

Table 26. rANCOVA of Vertical Activity, Female No Stress

rANCOVA of Vertical Activity, Female No Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	590410.852	1	590410.852	3.570	.081	.215	.417
Time * BLVA	304977.344	1	304977.344	1.844	.198	.124	.242
Time * Nic	398309.454	1	398309.454	2.409	.145	.156	.301
Error(Time)	2149797.843	13	165369.065				

rANCOVA of Vertical Activity, Female No Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	2408563.931	1	2408563.931	6.127	.028	.320	.629
BLVA	1123516.983	1	1123516.983	2.858	.115	.180	.347
Nic	40111.836	1	40111.836	.102	.754	.008	.060
Error	5110531.954	13	393117.843				

Table 27. rANCOVA of Vertical Activity, Female Stress

rANCOVA of Vertical Activity, Female Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	152031.066	1	152031.066	2.027	.178	.135	.261
Time * BLVA	1921.672	1	1921.672	.026	.875	.002	.053
Time * Nic	204394.914	1	204394.914	2.725	.123	.173	.334
Error(Time)	975259.203	13	75019.939				

rANCOVA of Vertical Activity, Female Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	637007.507	1	637007.507	1.421	.255	.099	.197
BLVA	1754641.149	1	1754641.149	3.914	.069	.231	.449
Nic	36848.409	1	36848.409	.082	.779	.006	.058
Error	5828573.226	13	448351.787				

Table 28. ANOVA of Vertical Activity, Male No Stress, Baseline

ANCOVA of Vertical Activity, Male No Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	539857.563	1	539857.563	3.457	.084	.198	.410
Intercept	23076014.063	1	23076014.063	147.759	.000	.913	1.000
Nic	539857.563	1	539857.563	3.457	.084	.198	.410
Error	2186425.375	14	156173.241				
Total	25802297.000	16					
Corrected Total	2726282.938	15					

Table 29. ANOVA of Vertical Activity, Male Stress, Baseline

ANCOVA of Vertical Activity, Male Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	138942.563	1	138942.563	.890	.362	.060	.142
Intercept	23148126.563	1	23148126.563	148.258	.000	.914	1.000
Nic	138942.563	1	138942.563	.890	.362	.060	.142
Error	2185871.875	14	156133.705				
Total	25472941.000	16					
Corrected Total	2324814.438	15					

Table 30. ANOVA of Vertical Activity, Female No Stress, Baseline

ANCOVA of Vertical Activity, Female No Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	663003.063	1	663003.063	2.930	.109	.173	.358
Intercept	34184485.563	1	34184485.563	151.050	.000	.915	1.000
Nic	663003.063	1	663003.063	2.930	.109	.173	.358
Error	3168376.375	14	226312.598				
Total	38015865.000	16					
Corrected Total	3831379.438	15					

Table 31. ANOVA of Vertical Activity, Female Stress, Baseline

ANCOVA of Vertical Activity, Female Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	43160.062	1	43160.062	.349	.564	.024	.085
Intercept	31880139.063	1	31880139.063	257.899	.000	.949	1.000
Nic	43160.063	1	43160.063	.349	.564	.024	.085
Error	1730607.875	14	123614.848				
Total	33653907.000	16					
Corrected Total	1773767.938	15					

Table 32. ANCOVA of Vertical Activity, Male No Stress, T1

ANCOVA of Vertical Activity, Male No Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	2153417.024	2	1076708.512	4.594	.031	.414	.672
Intercept	421281.554	1	421281.554	1.797	.203	.121	.237
BLVA	1711192.024	1	1711192.024	7.301	.018	.360	.705
Nic	180.147	1	180.147	.001	.978	.000	.050
Error	3047006.976	13	234385.152				
Total	46880360.000	16					
Corrected Total	5200424.000	15					

Table 33. ANCOVA of Vertical Activity, Male Stress, T1

ANCOVA of Vertical Activity, Male Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1116424.825	2	558212.412	1.945	.182	.230	.330
Intercept	616927.640	1	616927.640	2.150	.166	.142	.274
BLVA	1086495.825	1	1086495.825	3.786	.074	.226	.437
Nic	178567.981	1	178567.981	.622	.444	.046	.113
Error	3730573.175	13	286967.167				
Total	41643354.000	16					
Corrected Total	4846998.000	15					

Table 34. ANCOVA of Vertical Activity, Female No Stress, T1

ANCOVA of Vertical Activity, Female No Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	2033200.179	2	1016600.090	5.829	.016	.473	.779
Intercept	306993.248	1	306993.248	1.760	.207	.119	.233
BLVA	1299607.929	1	1299607.929	7.452	.017	.364	.714
Nic	92810.789	1	92810.789	.532	.479	.039	.104
Error	2267188.821	13	174399.140				
Total	36188998.000	16					
Corrected Total	4300389.000	15					

Table 35. ANCOVA of Vertical Activity, Female Stress, T1

ANCOVA of Vertical Activity, Female Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1031367.054	2	515683.527	2.796	.098	.301	.454
Intercept	83320.105	1	83320.105	.452	.513	.034	.096
BLVA	936348.992	1	936348.992	5.076	.042	.281	.550
Nic	207406.611	1	207406.611	1.124	.308	.080	.166
Error	2398020.383	13	184463.106				
Total	32862725.000	16					
Corrected Total	3429387.438	15					

Table 36. ANCOVA of Vertical Activity, Male No Stress, T2

ANCOVA of Vertical Activity, Male No Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1241338.272	2	620669.136	1.999	.175	.235	.338
Intercept	1072808.113	1	1072808.113	3.454	.086	.210	.406
BLVA	1181680.210	1	1181680.210	3.805	.073	.226	.439
Nic	70223.485	1	70223.485	.226	.642	.017	.073
Error	4037256.665	13	310558.205				
Total	55012825.000	16					
Corrected Total	5278594.938	15					

Table 37. ANCOVA of Vertical Activity, Male Stress, T2

ANCOVA of Vertical Activity, Male Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1906864.554	2	953432.277	5.485	.019	.458	.753
Intercept	936951.672	1	936951.672	5.390	.037	.293	.575
BLVA	1372137.992	1	1372137.992	7.893	.015	.378	.738
Nic	990877.729	1	990877.729	5.700	.033	.305	.598
Error	2259901.883	13	173838.606				
Total	54679769.000	16					
Corrected Total	4166766.438	15					

Table 38. ANCOVA of Vertical Activity, Female No Stress, T2

ANCOVA of Vertical Activity, Female No Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	361451.461	2	180725.730	.471	.635	.068	.111
Intercept	2691981.536	1	2691981.536	7.009	.020	.350	.687
BLVA	128886.398	1	128886.398	.336	.572	.025	.084
Nic	345610.501	1	345610.501	.900	.360	.065	.142
Error	4993140.977	13	384087.767				
Total	51768155.000	16					
Corrected Total	5354592.438	15					

Table 39. ANCOVA of Vertical Activity, Female Stress, T2

ANCOVA of Vertical Activity, Female Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	928619.391	2	464309.696	1.370	.288	.174	.243
Intercept	705718.468	1	705718.468	2.082	.173	.138	.267
BLVA	820213.829	1	820213.829	2.420	.144	.157	.302
Nic	33836.712	1	33836.712	.100	.757	.008	.060
Error	4405812.046	13	338908.619				
Total	62931147.000	16					
Corrected Total	5334431.438	15					

Table 40. Overall rANCOVA of Center Time

rANCOVA of Center Time Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta	Observed Power
Time	186.119	1	186.119	2.728	.104	.047	.368
Time * BLRt	196.096	1	196.096	2.874	.096	.050	.384
Time * Sex	10.099	1	10.099	.148	.702	.003	.067
Time * Stress	.274	1	.274	.004	.950	.000	.050
Time * Nic	32.779	1	32.779	.480	.491	.009	.105
Time * Sex * Stress	44.133	1	44.133	.647	.425	.012	.124
Time * Sex * Nic	1.957	1	1.957	.029	.866	.001	.053
Time * Stress * Nic	.874	1	.874	.013	.910	.000	.051
Time * Sex * Stress * Nic	21.393	1	21.393	.314	.578	.006	.085
Error(Time)	3752.402	55	68.225				

rANCOVA of Center Time Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	449.047	1	449.047	5.334	.025	.088	.621
BLRt	1755.356	1	1755.356	20.851	.000	.275	.994
Sex	120.361	1	120.361	1.430	.237	.025	.217
Stress	224.033	1	224.033	2.661	.109	.046	.361
Nic	3.604	1	3.604	.043	.837	.001	.055
Sex * Stress	62.899	1	62.899	.747	.391	.013	.136
Sex * Nic	21.474	1	21.474	.255	.616	.005	.079
Stress * Nic	.732	1	.732	.009	.926	.000	.051
Sex * Stress * Nic	25.538	1	25.538	.303	.584	.005	.084
Error	4630.254	55	84.186				

Table 41. rANCOVA of Center Time, Males

rANCOVA of Center Time Male, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	17.075	1	17.075	1.235	.276	.044	.188
Time * BLRt	19.740	1	19.740	1.427	.243	.050	.211
Time * Stress	10.347	1	10.347	.748	.395	.027	.133
Time * Nic	4.315	1	4.315	.312	.581	.011	.084
Time * Stress * Nic	44.231	1	44.231	3.198	.085	.106	.407
Error(Time)	373.414	27	13.830				

rANCOVA of Center Time Male, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	293.618	1	293.618	5.596	.025	.172	.626
BLRt	553.941	1	553.941	10.558	.003	.281	.879
Stress	37.671	1	37.671	.718	.404	.026	.129
Nic	2.351	1	2.351	.045	.834	.002	.055
Stress * Nic	8.656	1	8.656	.165	.688	.006	.068
Error	1416.627	27	52.468				

Table 42. rANCOVA of Center Time, Females

rANCOVA of Center Time Female, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	551.330	1	551.330	5.018	.034	.157	.579
Time * BLRt	588.973	1	588.973	5.361	.028	.166	.607
Time * Stress	5.182	1	5.182	.047	.830	.002	.055
Time * Nic	8.738	1	8.738	.080	.780	.003	.059
Time * Stress * Nic	5.436	1	5.436	.049	.826	.002	.055
Error(Time)	2966.371	27	109.866				

rANCOVA of Center Time Female, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	162.553	1	162.553	1.398	.247	.049	.207
BLRt	1276.251	1	1276.251	10.978	.003	.289	.891
Stress	155.196	1	155.196	1.335	.258	.047	.200
Nic	29.808	1	29.808	.256	.617	.009	.078
Stress * Nic	9.684	1	9.684	.083	.775	.003	.059
Error	3138.791	27	116.252				

Table 43. rANCOVA of Center Time, Male No Stress

rANCOVA of Center Time, Male No Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	4.884	1	4.884	.250	.625	.019	.075
Time * BLRt	13.513	1	13.513	.693	.420	.051	.121
Time * Nic	10.944	1	10.944	.561	.467	.041	.107
Error(Time)	253.583	13	19.506				

rANCOVA of Center Time, Male No Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	88.916	1	88.916	1.660	.220	.113	.223
BLRt	511.474	1	511.474	9.546	.009	.423	.815
Nic	18.807	1	18.807	.351	.564	.026	.085
Error	696.531	13	53.579				

Table 44. rANCOVA of Center Time, Male Stress

rANCOVA of Center Time, Male, Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	12.986	1	12.986	1.412	.256	.098	.197
Time * BLRt	6.517	1	6.517	.709	.415	.052	.122
Time * Nic	37.853	1	37.853	4.117	.063	.241	.468
Error(Time)	119.540	13	9.195				

rANCOVA of Center Time, Male Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	233.889	1	233.889	4.589	.052	.261	.509
BLRt	99.919	1	99.919	1.960	.185	.131	.255
Nic	.065	1	.065	.001	.972	.000	.050
Error	662.644	13	50.973				

Table 45. rANCOVA of Center Time, Female No Stress

rANCOVA of Center Time, Female, No Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	509.836	1	509.836	2.419	.144	.157	.302
Time * BLRt	729.041	1	729.041	3.459	.086	.210	.406
Time * Nic	8.942	1	8.942	.042	.840	.003	.054
Error(Time)	2739.896	13	210.761				

rANCOVA of Center Time, Female No Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	42.466	1	42.466	.212	.653	.016	.071
BLRt	1526.154	1	1526.154	7.631	.016	.370	.724
Nic	49.648	1	49.648	.248	.627	.019	.075
Error	2600.013	13	200.001				

Table 46. rANCOVA of Center Time, Female Stress

rANCOVA of Center Time, Female, Stress, Within Subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	21.019	1	21.019	3.206	.097	.198	.382
Time * BLRt	1.187	1	1.187	.181	.677	.014	.068
Time * Nic	5.442	1	5.442	.830	.379	.060	.135
Error(Time)	85.220	13	6.555				

rANCOVA of Center Time, Female, Stress, Between Subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	297.772	1	297.772	13.791	.003	.515	.929
BLRt	8.188	1	8.188	.379	.549	.028	.088
Nic	.751	1	.751	.035	.855	.003	.053
Error	280.687	13	21.591				

Table 47. ANOVA of Center Time, Male No Stress, Baseline

ANCOVA of Center Time, Male No Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	21.437	1	21.437	.880	.364	.059	.141
Intercept	1414.136	1	1414.136	58.037	.000	.806	1.000
Nic	21.437	1	21.437	.880	.364	.059	.141
Error	341.123	14	24.366				
Total	1776.696	16					
Corrected Total	362.560	15					

Table 48. ANOVA of Center Time, Male Stress, Baseline

ANCOVA of Center Time, Male Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	5.085	1	5.085	.263	.616	.018	.077
Intercept	1085.703	1	1085.703	56.147	.000	.800	1.000
Nic	5.085	1	5.085	.263	.616	.018	.077
Error	270.717	14	19.337				
Total	1361.504	16					
Corrected Total	275.802	15					

Table 49. ANOVA of Center Time, Female No Stress, Baseline

ANCOVA of Center Time, Female No Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	8.18	1	8.180	.243	.630	.017	.075
Intercept	1964.706	1	1964.706	58.280	.000	.806	1.000
Nic	8.180	1	8.180	.243	.630	.017	.075
Error	471.961	14	33.711				
Total	2444.846	16					
Corrected Total	480.140	15					

Table 50. ANOVA of Center Time, Female Stress, Baseline

ANCOVA of Center Time, Female Stress, Baseline

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	4.601	1	4.601	.467	.506	.032	.098
Intercept	758.727	1	758.727	76.967	.000	.846	1.000
Nic	4.601	1	4.601	.467	.506	.032	.098
Error	138.009	14	9.858				
Total	901.337	16					
Corrected Total	142.610	15					

Table 51. ANCOVA of Center Time, Male No Stress, T1

ANCOVA of Center Time, Male No Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	186.157	2	93.078	3.714	.053	.364	.574
Intercept	67.740	1	67.740	2.703	.124	.172	.331
BLRt	179.358	1	179.358	7.156	.019	.355	.696
Nic	.529	1	.529	.021	.887	.002	.052
Error	325.822	13	25.063				
Total	2622.233	16					
Corrected Total	511.979	15					

Table 52. ANCOVA of Center Time, Male Stress, T1

ANCOVA of Center Time, Male Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	52.005	2	26.002	.806	.468	.110	.159
Intercept	178.549	1	178.549	5.534	.035	.299	.586
BLRt	27.700	1	27.700	.859	.371	.062	.138
Nic	17.386	1	17.386	.539	.476	.040	.105
Error	419.402	13	32.262				
Total	2107.609	16					
Corrected Total	471.407	15					

Table 53. ANCOVA of Center Time, Female No Stress, T1

ANCOVA of Center Time, Female No Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	2193.021	2	1096.511	5.152	.023	.442	.724
Intercept	129.009	1	129.009	.606	.450	.045	.112
BLRt	2182.410	1	2182.410	10.254	.007	.441	.841
Nic	8.225	1	8.225	.039	.847	.003	.054
Error	2766.970	13	212.844				
Total	9791.284	16					
Corrected Total	4959.991	15					

Table 54. ANCOVA of Center Time, Female Stress, T1

ANCOVA of Center Time, Female Stress, T1

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	15.7	2	7.850	1.408	.279	.178	.249
Intercept	80.283	1	80.283	14.404	.002	.526	.939
BLRt	7.804	1	7.804	1.400	.258	.097	.195
Nic	5.119	1	5.119	.918	.355	.066	.144
Error	72.458	13	5.574				
Total	951.931	16					
Corrected Total	88.159	15					

Table 55. ANCOVA of Center Time, Male No Stress, T2

ANCOVA of Center Time, Male No Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	346.462	2	173.231	3.607	.057	.357	.561
Intercept	26.060	1	26.060	.543	.474	.040	.105
BLRt	345.630	1	345.630	7.197	.019	.356	.699
Nic	29.222	1	29.222	.609	.449	.045	.112
Error	624.291	13	48.022				
Total	3414.325	16					
Corrected Total	970.753	15					

Table 56. ANCOVA of Center Time, Male Stress, T2

ANCOVA of Center Time, Male Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	90.01	2	45.005	1.613	.237	.199	.280
Intercept	68.326	1	68.326	2.448	.142	.158	.305
BLRt	78.737	1	78.737	2.821	.117	.178	.343
Nic	20.532	1	20.532	.736	.407	.054	.125
Error	362.782	13	27.906				
Total	1768.486	16					
Corrected Total	452.791	15					

Table 57. ANCOVA of Center Time, Female No Stress, T2

ANCOVA of Center Time, Female No Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	109.206	2	54.603	.276	.763	.041	.085
Intercept	423.293	1	423.293	2.139	.167	.141	.273
BLRt	72.785	1	72.785	.368	.555	.028	.087
Nic	50.366	1	50.366	.254	.622	.019	.075
Error	2572.939	13	197.918				
Total	6798.010	16					
Corrected Total	2682.146	15					

Table 58. ANCOVA of Center Time, Female Stress, T2

ANCOVA of Center Time, Female Stress, T2

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	2.251	2	1.125	.050	.952	.008	.056
Intercept	238.508	1	238.508	10.566	.006	.448	.852
BLRt	1.570	1	1.570	.070	.796	.005	.057
Nic	1.075	1	1.075	.048	.831	.004	.055
Error	293.449	13	22.573				
Total	2085.413	16					
Corrected Total	295.700	15					

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